Innovations in Food Ingredients
&
Food Safety

PROCEEDINGS OF

THE FIRST INTERNATIONAL CONFERENCE

Editors
Anil Kumar Anal
Gerhard Schleining

12-13 September 2018
Bangkok, Thailand

Co-funded by the
Erasmus+ Programme
of the European Union

The 1st International Conference on "Innovations in Food Ingredients & Food Safety (IFIFS 2018)"

Organized by

Asian Institute of Technology (AIT), Thailand

ISEKI-Food Association (IFA), Austria

12-13 September 2018
Bangkok, Thailand

All papers accepted for the Proceedings have been *Peer Reviewed* by the Conference Scientific Committee members. The papers have been presented during the First International Conference on Innovations in Food Ingredients and Food Safety (IFIFS 2018), held during 12-13 September 2018 in Bangkok, Thailand.

Co-funded by the
Erasmus+ Programme
of the European Union

Published by:

Asian Institute of Technology, Klong Luang, PO Box 4, Pathumthani 12120, Thailand

ISBN 978-974-8257-97-6

PREFACE

The global food system today is beset by serious challenges and risks. Food demand is on rise due to population growth and changing consumption patterns; production and prices have become more volatile; hunger and poverty levels remain high and unsustainable practices exacerbate environmental challenges. Food produce is increasingly called upon to address a wide range of critical needs; safe and nutritious food for nine billion people by 2050; and higher and more resilient incomes for livelihoods. Though food-processing industries have grown tremendously, food safety and quality remain always the prime concerns of consumers and food processing industries. Unsafe food causes both acute and chronic illness, and reduces the bioavailability of nutrients, particularly for vulnerable consumers. An estimated 600 million (almost 1 in 10) people in the world fall ill after eating contaminated food and 420 000 die every year. Diarrheal diseases are the most common illnesses resulting from the consumption of contaminated food, causing 550 million people to fall ill and 230 000 deaths every year. Food-borne diseases impose a heavy social and economic burden on communities, especially affecting their health care systems, and economic productivity. Implementation of food safety standards is important for the food industry in order to meet the consumer expectations. Changes in food consumption patterns and the growth of international megabrands have put pressure on the food industry, especially in new product development (NPD). The industry's approach to NPD stems primarily from innovation sourced from either mergers and acquisitions or multinationals for products, or from suppliers for processes. With the variation across segments appearing to be competitive, it is crucial for manufacturers to understand that success is not only about marketing efforts or the quality of a product, but also by the brand's capability to observe current trends in order for its products to reach potential consumers.

The aim of this International Conference is to bring together researchers, industries and policy developers to discuss on challenges, trends and innovative approaches for safer and sustainable food and beverage produce. The 1st day is dedicated to disseminate outcomes of 2 ERASMUS+ capacity building projects (SEA-ABT and ASIFOOD), which are related to food quality and safety of food and beverages and to a burning issue and emerging topic on sugar and sweeteners. The 2nd day will focus on timely and trending topics that address the opportunities and challenges in the emerging innovative technology for sustainable and safer production of food and beverages.

Anil Kumar Anal
Gerhard Schleining

ORGANISING COMMITTEE

Anil KUMAR ANAL, Asian Institute of Technology (AIT), Thailand

Gerhard SCHLEINING, ISEKI-Food Association (IFA), Austria

Anita HABERSHUBER, ISEKI-Food Association (IFA), Austria

Manisha SINGH, Asian Institute of Technology (AIT), Thailand

SCIENTIFIC COMMITTEE

Anil KUMAR ANAL, Asian Institute of Technology (AIT), Thailand - **Chair**

Gerhard SCHLEINING, ISEKI-Food Association (IFA), Austria - **Chair**

Sylvie AVALLONE, Montpellier SupAgro Higher School of Education, France

Chaleeda BOROMPICHAICHARTKUL, Chulalongkorn University (CU), Thailand

Dario COMPAGNONE, University of Teramo (UNITE), Italy

Kriskamol NA JOM, Kasetsart University (KU), Thailand

Thi Minh Tu NGUYEN, Hanoi University of Science and Technology (HUST), Vietnam

Navaphattra NUNAK, King Mongkut University (KMITL), Thailand

Paola PITTIA, University of Teramo (UNITE), Italy

Marie-Louise SCIPPO, University of Liège, Belgium

Sarn SETTACHAIMONGKON, Chulalongkorn University (CU), Thailand

Suesut TAWEEPOL, King Mongkut University (KMITL), Thailand

Sasitorn TONGCHITPAKDEE, Kasetsart University (KU), Thailand

Yves WACHE, L'Institut agronomique, vétérinaire et forestier de France (IAVFF), France

Ulaiwan WITHAYAGIAT, Kasetsart University (KU), Thailand

CONTENTS

PREFACE.....v

ORGANISING COMMITTEE.....vi

SCIENTIFIC COMMITTEE.....vi

Effect of Disaccharides on the Physical Properties of Rice Starch Edible Film

Thongkorn Ployetchara, Shoichi Gohtani..... 1

Sequential Extraction of Hyaluronic Acid and Collagen from Chicken Eggshell Membrane

Nina Karla Alparce, Athapol Noomhorm, Anil Kumar Anal 13

Quality Evaluation of Maltose Syrup Produced from Hydrolysis of Cassava (*Manihot esculenta* Crantz) Starch Using Malted Rice (*Oryza sativa* L.)

Theavy Srey, Erlinda I. Dizon, Katherine Ann C. Israel, Leoncio C. Raymundo, Wilma A. Hurtada, Edralina P. Serrano.....29

Extraction and *in vitro* evaluation of antidiabetic efficacy of bioactive extracts from okra (*Abelmoschus esculentus)*

Manisha Anand, Smriti Shrestha, Manisha Singh and Anil Kumar Anal.....43

The Use of Predictive Models in the Context of Food Spoilage: The Case of White Pudding

Emilie Cauchie, Nicolas Korsak, Mariem Ellouze, Laurent Delhalle, Bernard Taminiau, Fall Papa Abdoulaye and Georges Daube 51

Effects of Drying Temperature on Color and Total Curcuminoids Contents in Turmeric

Nilobon Komonsing, Pramote Khuwijitjaru, Marcus Nagle, Busarakorn Mahayothee, Joachim Mueller.....61

Simultanious Vacuum Falling Film and Rotary Evaporation for Producing Sweet Sorghum Syrup

N. Istianah, S.S.Yuwono, D.Y. Ali, R.J.A. Aghata 71

Detection of 3-monochloropropane-1,2-diol (3-MCPD) Using Molecularly Imprinted Poly(3-aminophenylboronic acid)

Niroshan Chinthaka Gunawardena, Loc Thai Nguyen.....77

A NMR-Based Metabolomics Approach for Reducing Food Losses: The Example of Minced Pork Meat

Emilie Cauchie, Nicolas Korsak, Justine Leenders, Ghislain Baré, Assia Tahiri, Laurent Delhalle, Pascal de Tullio and Daube Georges 89

Development and Evaluation of W/O/W Emulsion Stabilized by Polysaccharide-Protein Based Interaction

Mishenki Rajapakse, Anil K. Anal 101

Box-Behnken Design Based Mango (Mangifera indica) Wine Preparation, Screening of Phytochemicals and Antioxidant Activity by DPPH Radical Scavenging Assay

Win Nandar Thet, Theint Theint Hlaing, Su Lwin Htike 113

Investigation of Phytochemicals, Antioxidants and Antimicrobial Activities in Sacha Inchi (*Plukenetia volubilis*) Press Cake of Myanmar Origin

Su Lwin Htike, Si Si Win , Win Nandar Thet 123

Optimization of Lactic Acid Production from Fermentation of Vegetable Wastes through Response Surface Method

Shwe Zin Moe, Si Si Win..... 131

Recovery and Functional Properties of Muscle Proteins Extracted from Indian Threadfin Bream (*Nemipterus Japonicus*) by pH-Shift Processing

Supriya. D. Meshre, S. B. Patange, Rakesh R. Jadhav and Sanna Shah..... 139

Effect of Growing Culture on Growth Characteristics of *Acetobacter Pasteurianus* NH6 and Their Survival after Heat-Pump Drying

Hien Nguyen Minh and Huan Phan Tai..... 149

Studies on Traditional Making Process, Compositional Analysis and Microbial Population of Myanmar Traditional Tea Leaves, *Laphet*

Hnin Htet Htet Shein, Kyaw Nyein Aye 155

Studies on Doxycycline Degradation Fraction in Milk Curd and Whey by Plant Rennet

Aye Moe Thwe, Swe Swe Win 161

Determination of Total Fat and Iodine Value in Glutinous Rice-Based Oily Snacks (*Htoe Mont*)

Aye Thinzar Khine, Myint Myint 169

Effect of Milling on Arsenic Contents in Long-Grain Rice

Yaowalak Rooppat, Wenika Benjapong, Jintana Sirivararsenicai, Rodjana Chunhabundit, Weeraya Karnpanit, Sirirat Khundam, Jakkit Sakolkittinapakul 175

Effect of Boiling on Arsenic Contents and Antioxidant Activity in Thai Rice Noodle Product Incorporating Pigmented Broken Rice and Rice Bran

Sirirat Khundam, Wenika Benjapong, Pornrat Sinchaipanit, Ratchanee Kongkachuichai, Weeraya Karnpanit, Yaowalak Rooppat, Jakkit Sakolkittinapakul..... 183

Inadequate intake of Dietary Fiber, Vitamin B_{12}, and Calcium in Thai Elders

Budtree Treesattayakul, Thunnalin Winuprasith, Budsakorn Teeranurak, Dunyaporn Trachootham..... 193

Identification of Bacteria Isolated from the Food Contact Surfaces in Poultry Processing Plant

Pawarisa Boonkoy, Warapa Mahakarnchanakul..... 205

Impact Assessment of Fiber Rich Supplement on Blood Glucose Level of Diabetic Subjects

Seema Medhe, Asha Arya, Mayuri Choudhary..... 215

ANNEXES..... 221

ANNEX-1: CONFERENCE TOPICS..... 222

ANNEX-2: AGENDA..... 223

ANNEX-3: ORGANISING INSTITUTIONS..... 226

ANNEX-4: SPONSORS..... 227

Effect of Disaccharides on the Physical Properties of Rice Starch Edible Film

Thongkorn Ployetchara[1], Shoichi Gohtani[2]*

[1]Department of Applied Bioresource Science (Food Science), The United Graduate School of Agricultural Sciences, Ehime University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795 Japan
[2]Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795 Japan
*Corresponding Author (gohtani@ag.kagawa-u.ac.jp) Tel. /Fax: +81878 913103.
Additional corresponding Author (mo_2u@hotmail.com)

ABSTRACT

The effect of the concentration of some disaccharides (sucrose, maltose, and trehalose) on the physical properties of rice starch-based edible film was studied. Samples were prepared containing 3% (w/w) starch with 0, 10, 20, and 30% (w/w) disaccharide added to the starch as a plasticizer. The thickness, moisture content, and water solubility of the film increased, while the transparency of the film decreased with an increase in the concentration of all three disaccharides in the present study. The mechanical properties of the film with and without disaccharide showed different results during storage for 4 weeks. The breaking stress of the film without disaccharide increased and the breaking strain decreased with an increase in storage time. Disaccharide decreased the breaking stress of the film with an increase in storage time. The breaking strain of the films with 10% disaccharide was higher than that of the film without disaccharide. The breaking strain of the films with 20% of all three disaccharides and 30% sucrose and trehalose were not different to that of the film without disaccharide, while that of the film with 30% maltose was less than that of the film without disaccharide on the day of preparation. The decrease in breaking strain due to storage became progressively greater with an increase in concentration of all three disaccharides. X-ray diffraction showed the relative crystallinity of the film increased with an increase in concentration of sucrose and decreased with an increase in concentration of maltose and trehalose on the day of preparation. After storage, the intensity and relative crystallinity increased with an increase in concentration of all three disaccharides. Moreover, relative crystallinity increased in the order of sucrose, maltose, and trehalose. Pearson bivariate correlation showed high correlation between the concentration of disaccharide and some properties of the film: thickness, transparency, moisture content, water solubility, and mechanical properties. Adding disaccharide as a plasticizer affected the properties of the rice starch film by concentration and type of disaccharide because of the hydroxyl groups of the disaccharide.

KEYWORDS: Starch Film, Disaccharide, Mechanical Properties, Recrystallization

1 INTRODUCTION

Edible film is food packaging that can be used instead of plastic and has advantages for the environment. Starch, a polymer composed of amylose and amylopectin, is a material used for producing biodegradable and edible films because of its low cost. Various sources of starch are selected for properties such as amylose-amylopectin content and granule size that affect the properties of film (Galdeano *et al.*, 2009). Numerous studies have reported that starch film has good mechanical strength and is a good barrier (Apriyana *et al.*, 2016; Tongdeesoontorn *et al*., 2011). Also, rice starch could be applied to many food products, and producing edible film from rice starch and flour has been reported (Dias et al., 2010). Plasticizers are an additive used to enhance the physical and mechanical properties of edible film to produce a flexible and more effective film because of the reduced interactions between starch molecules (Dias *et al*., 2010; Espita *et al.*, 2014; Ghasemlou *et al.*, 2013; Skurtys *et al*., 2010; Zhang and Han, 2006).

Sugars, especially disaccharides such as sucrose and maltose, affect the rheological properties and recrystallization of the starch system (Smits *et al.,* 2003; Yoo and Yoo 2005). Sucrose has been reported to inhibit recrystallization, decrease tensile strength, and increase elongation at break (Piermaria *et al*., 2011; Souza *et al*., 2012; Veiga-Santos *et al*., 2007). Maltose and trehalose have been reported to disrupt starch chain interaction and prevent recrystallization in the starch system (Babić *et al*., 2009; Smits *et al.,* 2003; Zhang *et al*., 2017). The sugar affects the starch system by inhibiting starch chain mobility in the amorphous region and is reorganized by the hydroxyl (OH) groups of the sugar (Abu-Jdayil *et al.,* 2004; Ploypetchara *et al.,* 2015). Thus, the OH groups of the sugar are important for interaction in the matrix system and affect film properties. Many studies have reported on the hydrogen bonds and OH groups of additives that affect the matrix system (Tongdeesoontorn *et al.,* 2011). (Saberi *et al*., 2016) reported that a high number of OH groups promotes more hydrogen bonds formation in the film matrix and increases the moisture content in film. Also, (Smits *et al.,* 2003) reported on the number of OH groups in the plasticizer involved in the recrystallization of starch. In this work, we investigated the effect on film properties of both type and concentration of disaccharide, the number of OH groups, the number of equatorial OH groups and hydration number (Table 1).

Table 1: Properties of disaccharide

Sugar	Sucrose	Maltose	Trehalose
Monomers	Glucose-Fructose	Glucose- Glucose	Glucose-Glucose
OH groups	8	8	8
Equatorial OH groups	6.3	7.6	8
Hydration number	5	5.9	6.6

(Kawai *et al*., 1992; Ikeda *et al*., 2014; Saberi *et al.,* 2017)

2 MATERIAL AND METHODS

2.1 Materials

Raw starch used in this study was normal rice starch (Matsutani Co. Ld, Japan). Sugars used as plasticizer were sucrose, maltose monohydrate (Nacalai Tesque, Inc., Kyoto, Japan), and trehalose (Hayashibara Co., Ltd., Japan). Pure water was produced by Barnstead (Barnstead International, USA).

2.2 Film Suspension Preparation and Film Formation

Film suspensions were prepared by adding 3% starch (w/w dry basis) to pure water and stirring for 15 min, then adding 10, 20, and 30% disaccharide as a plasticizer (sucrose, maltose, and trehalose) (w/w of starch weight) and stirring for 15 min. After that, the samples were heated at 95 °C for 30 min in a water bath. Bubbles in the suspensions were reduced by vacuum, and then the suspension was allowed to cool to room temperature to cast film. Films were prepared from the film suspensions by drying 8 g of film suspension in Petri dishes (7.7 cm diameter). The film suspensions were dried at 50 °C in a circulated air drying oven (OFX-50; As One, China) for around 1.5 h, and then the dried films were maintained at 25 °C in a cool incubator (CN-25B; Mitsubishi, Japan) overnight before removing from the Petri dishes. The film samples were stored in plastic bags under in a dry atmosphere with silica gel in desiccators at 25 °C until measurement (Sanyang *et al*., 2015).

2.2 Film Thickness

Film thickness was measured with a micrometer (Niigata Seiki, Japan), and the thickness at 10 random positions of the film was averaged (Rodríguez *et al*., 2006).

2.3 Film Transparency

Film transparency (TR) was measured using a JASCO V-600 spectrophotometer. Film samples were cut to 10 × 40 mm and placed into the spectrophotometer cell. Transmission was measured at 560 nm and TR calculated using the following equation:

$$TR = \log T/x \quad (1)$$

Where, T is transmission (%) and x is thickness of the film (mm) (Saberi *et al.*, 2016).

2.4 Film Moisture Content and Water Solubility

To determine the moisture content of the film sample (MC), the sample was dried in an oven (SDW 27; Sanyo, Japan) at 105 °C until constant weight was reached. The moisture content was calculated using the following equation:

$$MC\ (\%) = (W_1-W_2)\times 100 / W_1 \quad (2)$$

Where, W_1 is the weight of the film sample before drying and W_2 is the weight of the film sample after drying when constant weight is reached.

To measure the water solubility of the film sample, the film was cut to 40 × 15 mm and dried. The dried film sample was then immersed in 50 ml of distilled water for 24 h and filtered. The residue after filtering was dried at 110 °C until constant weight was reached. Water solubility was calculated using the following equation (Farahnaky *et al.*, 2013):

$$\text{Water solubility (\%)} = (\text{initial dry weight} - \text{final dry weight}) \times 100 / \text{initial dry weight} \quad (3)$$

2.5 Mechanical Properties

Breaking stress and breaking strain were determined by using a Rheoner II Creep meter RE2-33005B (Yamaden, Japan) with a 20 N load cell. Film samples were cut to 5 × 15 mm length and subjected to a tensile test at a crosshead speed of 1 mm/s (Saberi *et al.*, 2016). The breaking stress and breaking strain of the film samples were measured on the day of preparation (day 0) and after storage for 1 week and 4 weeks.

2.6 Film Crystallinity

X-ray diffraction patterns of the starch films were determined by using Nano-viewer (Rigaku, Japan) equipped with a PILATUS at an applied voltage and filament current of 40 kV and 30 mA, respectively. The wavelength of the radiation source was 0.154 nm. The diffraction angle (2θ) was from 0 to 27°. Crystallinity of the film was evaluated with relative crystallinity (%) calculated as:

$$\text{Relative crystallinity (\%)} = I_f / I_s \times 100 \quad (4)$$

Where, I_f is the integral of the crystalline area of the film sample on the x-ray diffraction pattern (peak at 15, 17, 18, 20, and 23°) and I_s is the integral of the crystalline area of the raw starch sample on the X-ray diffraction pattern (Primo-Martín *et al.*, 2007). The crystalline area was estimated using Igor Pro software (Multi-peak Fit version 2.0). The crystallinity of the film samples was measured on the day of preparation (day 0), and after storage for 1 week and 4 weeks.

2.7 Statistical Analysis

All the samples were analyzed statistically using SPSS version 17. Different means were investigated using ANOVA and Duncan's multiple range tests at a level of significance of 0.05.

Correlation of variables was determined using Pearson bivariate correlations at the 0.01 and 0.05 significance levels.

3 RESULTS AND DISCUSSION

3.1 Film Thickness

The thickness of the rice starch film increased significantly by adding and increasing the concentration of disaccharide (Table 2). Film thickness increased with an increase in plasticizer content, similar to the results in many other studies (Adjouman *et al.*, 2017; Laohakunjit and Noomhorm, 2004; Zhang and Han, 2006); Hafnimardiyanti and Armin, 2016; Adjouman *et al*., 2017). Film thickness increases because the plasticizer molecule disturbs the intermolecular linkage of the polymer chains resulting in an expanded structure, increased volume, and a restructuring of polymer film to be thicker (Razavi *et al.*, 2015). Moreover, the increase in solid content of the total mass in the film suspension by adding sugar could reorder the molecular chain to be more compact in the composite starch matrix (Apriyana *et al.*, 2016).

3.2 Film Transparency

Transparency is important in food packaging; a low transparency helps to protect food from light. Thus, lower transparency results in a higher quality food packaging film (Saberi *et al.*, 2016). In this work, the transparency of the rice starch edible film decreased with increasing disaccharide concentration for all three disaccharides tested in this study (Table 2). This result is similar to that of Hafnimardiyanti and Armin (2016) who found that plasticizer decreases transparency of mocaf flour film. Film transparency decreased with an increase in the number of OH groups because of sugar bridges crosslink formation between polymer chains, and the crosslinks of sugar bridges affect the refractive index to prevent light passing through the film matrix (Saberi *et al*., 2017). Film transparency also had a negative relationship with thickness (Table 3), which is similar to the results reported in many previous studies (Galdeano *et al.*, 2013; Piermaria et al., 2011; Saberi *et al.*, 2016). The difference in transparency from adding a different plasticizer is caused by the plasticizer having different molecular weight, composition, natural properties, and other characteristics (Saberi *et al.*, 2017).

Table 2: Properties of rice starch film plasticized by different disaccharide and concentration.

Disaccharide / Concentration (%)		Thickness (mm)	Transparency (%)	Moisture content (%)	Water solubility (%)
Control	0	0.0442 ± 0.002^{a}	36.90 ± 0.25^{g}	12.34 ± 0.97^{a}	14.74 ± 3.97^{a}
Sucrose	10	0.0527 ± 0.001^{b}	30.42 ± 0.24^{d}	27.52 ± 1.85^{c}	17.82 ± 3.88^{ab}
	20	0.0583 ± 0.002^{c}	27.18 ± 0.12^{bc}	28.34 ± 1.82^{d}	20.21 ± 3.09^{abc}
	30	0.0627 ± 0.000^{e}	25.40 ± 0.63^{a}	28.88 ± 2.24^{d}	29.48 ± 2.75^{ef}
Maltose	10	0.0523 ± 0.001^{b}	31.09 ± 0.12^{e}	24.75 ± 1.93^{b}	21.16 ± 6.42^{abcd}
	20	0.0599 ± 0.001^{cde}	27.09 ± 0.06^{bc}	24.88 ± 3.06^{bc}	24.30 ± 4.79^{bcde}
	30	0.0617 ± 0.001^{de}	26.51 ± 0.38^{b}	25.02 ± 1.80^{bc}	30.57 ± 5.05^{ef}
Trehalose	10	0.0503 ± 0.001^{b}	32.21 ± 0.01^{f}	28.14 ± 1.35^{d}	24.19 ± 4.77^{cde}
	20	0.0591 ± 0.002^{cd}	27.62 ± 0.37^{c}	27.91 ± 0.58^{d}	27.57 ± 2.19^{def}
	30	0.0632 ± 0.001^{e}	25.57 ± 0.12^{a}	30.03 ± 1.70^{d}	34.25 ± 5.55^{f}

*Different letters in each column indicates significant difference at $p \leq 0.05$.

3.3 Film Moisture Content

The moisture content of the rice starch film increased significantly ($p \leq 0.05$) with the addition of disaccharide (Table 2). Moisture content increases because of the hygroscopic nature of a plasticizer that increases water retention during the last period of film drying (Razavi *et al.,* 2015). The moisture content of the film in this study increased with an increase in plasticizer content because of the increasing hydrophilic matrix that absorbed moisture into the film. Moreover, plasticizer is more hygroscopic than starch and acts as a water holding agent; a higher plasticizer content results in a higher amount of water in starch-plasticized film and increases plasticized activity (Apriyana *et al.,* 2016; Adjouman *et al.,* 2017; Zhang and Han, 2006). Different types of plasticizer show different results because of differences in the number of OH groups (Hafnimardiyanti and Armin 2016). (Jatmiko *et al.,* 2016) reported that film with more plasticizer content absorbs more moisture, increasing the moisture absorption rate and moisture absorption capacity because of the increasing hydrophilicity of the film by the OH groups of the plasticizer.

3.4 Film Water Solubility

As shown in Table 2, the water solubility of the rice starch film significantly increased with the addition of all three disaccharides and an increase in the concentration of the disaccharides. Adding plasticizer increased the water solubility of the edible film because of the hydrophilic and hygroscopic properties of the film-forming compounds and based on the humidity of the starch film; plasticizer interacts with the film matrix and increases the space between the polymer chains resulting in water migrating into the film and increasing solubility (Laohakunjit and Noomhorm, 2004; Hafnimardiyanti and Armin 2016). Moreover, Table 2 shows water solubility tends to increase with an increasing number of equatorial OH groups of the disaccharide: trehalose > maltose > sucrose at 8, 7.6, and 6.3, respectively (Kawai *et al.,* 1992). A higher equatorial OH per molecule has a stronger stabilizing effect on the water structure, and a stronger interaction with water molecules by hydrogen bond due to the higher hydration number and bigger hydrate shell, resulting in higher water holding capacity (Russ *et al.,* 2014).

3.5 Mechanical Properties

The breaking stress of the rice starch film decreased with increased disaccharide concentration (Figure 1). The decrease in breaking stress with an increase in plasticizer content is due to the arrangement of the polymer chains and the hydrogen bonding being disturbed by the plasticizer; the rearrangement and the hydrogen bonds decrease the interaction and cohesiveness of the polymer chains and also affect the crystallinity and flexibility of film (Laohakunjit and Noomhorm, 2004). On the day of preparation, the breaking strain of the films with 10% disaccharide was higher than the film without disaccharide, the breaking strain of the films with 20% disaccharide and 30% sucrose and trehalose were not different to the film without disaccharide, and the breaking strain of the film with 30% maltose was less than that of the film without disaccharide (Figure 1). (Laohakunjit and Noomhorm, 2004) reported that the elongation of rice starch film plasticized with sorbitol increases with increasing concentration more than that with glycerol and elongation of film decreases using glycerol at 35% concentration.

At a low concentration of added plasticizer, the breaking strain increases because the plasticizer replaces the intermolecular bond between amylose, amylopectin, and amylose-amylopectin of the starch with the hydrogen bond of the starch-plasticizer molecule, and the plasticizer reduces starch chain rigidity and promotes chain mobility of flexible film (Laohakunjit and Noomhorm, 2004; Sanyang et al., 2015).

The breaking strain in this study decreased with 20 and 30% added disaccharide because of the anti-plasticizing effect at a high concentration of plasticizer. The strong interaction between plasticizer and biopolymer might result in loss of macromolecular mobility; a higher number of OH groups is more hydrophilic and results in a more efficient plasticizing agent, and therefore, the film would be too brittle and too tacky (Apriyana *et al.,* 2016; Sanyang *et al.,* 2015; Souza *et al*., 2012). Moreover, when the

level of plasticizer content is more than the level suitable for compatibility with starch, the matrix system undergoes phase separation (Sanyang *et al.*, 2015).

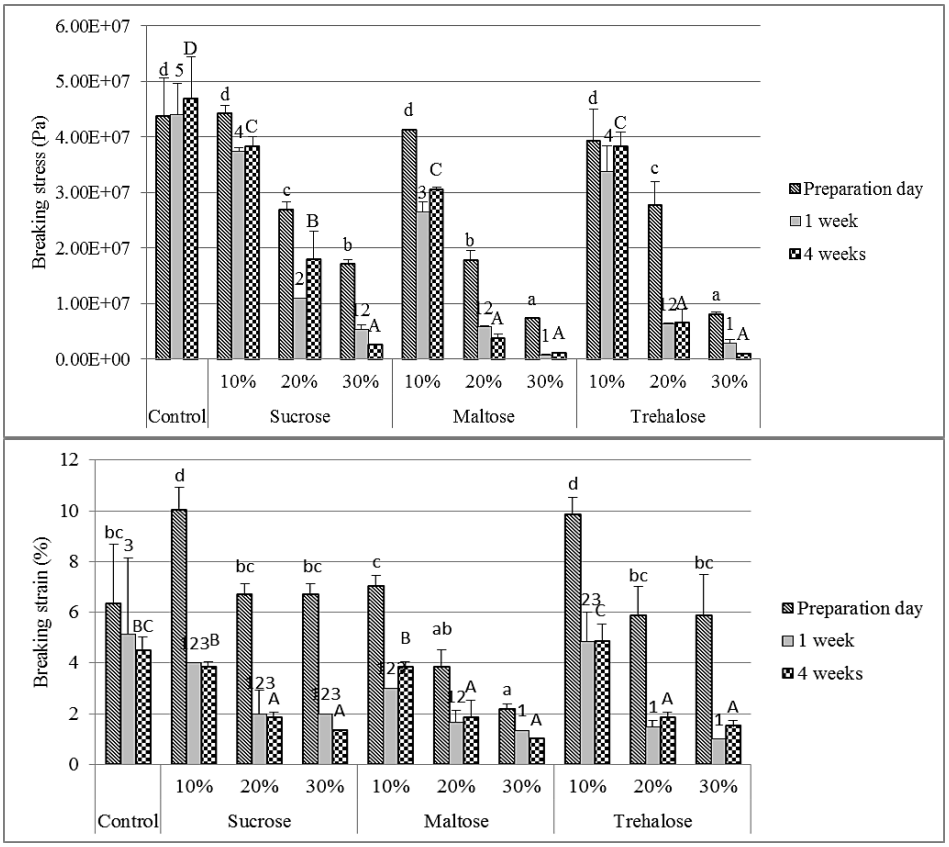


Figure 1: Breaking stress and breaking strain of rice starch film plasticized by disaccharide
*Different small letters in each column indicates difference on the day of preparation, with significant difference at p≤0.05.
**Different number letters in each column indicates difference at 1 week, with significant difference at p≤0.05.
***Different capital letters in each column indicates difference at 4 weeks, with significant difference at p≤0.05.

Nindjin *et al.*, (2015) reported similar results in that tensile strength and breaking strain decrease with a high of sucrose content (15–20%) added to cassava starch film because plasticization decreases the intermolecular force between the polymer chains, and the film matrix becomes less dense and less facilitating of polymer chains, resulting in a decrease in film resistance. Sucrose might also decrease elongation at break by a monomer structure composed of a bulky ring structure such as α-D-glucose and β-D-fructose, and the interaction between the sugar ring chain and starch polymeric chain could be more difficult than that of a straight sugar chain and starch polymer (Nindjin *et al.*, 2015).

During storage, the rice starch film without disaccharide showed increased breaking stress and decreased breaking strain, while the breaking stress of the film containing disaccharide decreased with an increase in concentration of the disaccharide. Breaking strain decreased with storage time in this study because of the anti-plasticization effect of added sugar. After storage, the resistance of a film matrix increases and stretchability decreases because of microstructural reorganization, starch retrogradation and expansion of a proportion of the crystalline zones (Apriyana *et al.*, 2016).

3.6 Film Crystallinity

The x-ray diffraction pattern of the rice starch film showed peaks at 15, 17, 20, and 23° (Figure 2), and the intensity of the crystal peak and relative crystallinity increased with an increase in storage time. On the day of preparation, the intensity showed little difference between concentration and type of disaccharide. The relative crystallinity of the rice starch film with added sucrose increased with

increasing concentration, similar to the results of Apriyana *et al.*, (2016) who showed that crystallinity increases with increased glycerol content because of increased macromolecular mobility, in which a microcrystalline junction forms and results in re-crystallization However, relative crystallinity decreased with an increasing concentration of maltose and trehalose in this study because of an increase in moisture content, similar to the result of (Famá *et al.*, 2005) and (Sanyang *et al.*, 2016) who reported that adding plasticizer inhibits crystal growth caused by interference to amylose packing and chain attraction or because of the increase in moisture content from the constant water activity.

After storage, the intensity and relative crystallinity increased with the increasing concentration of all three of disaccharides (sucrose, maltose, and trehalose). In the amorphous matrix, sugar delay the crystallization process through the interaction with starch, but the amorphous sugar has hygroscopic properties and adsorbs water from its surroundings as a result of the crystallization still progressing during storage (Buera *et al.*, 2005). The increasing in relative crystallinity was retarded in the order of trehalose, maltose, and sucrose based on the number of equatorial OH groups, which on trehalose was more than that of maltose and sucrose, similar to the result of (Zhang *et al.*, 2017) who found that trehalose retards tapioca starch gel retrogradation more than sucrose. (Smits *et al.*, 2003) reported that a macromolecular plasticizer or high number of OH groups could interact with ghost structures of starch and hinder interaction with polysaccharide chains; recrystallization increased prevention by reducing crystallization of the remaining ghost structures. A high number of OH groups strongly inhibits starch recrystallization because the OH groups have a hydrophilic property and the interaction of the hydrogen bonds with water increases; thus, a higher number of OH groups results in greater hydrogen bond interaction and higher inhibition starch recrystallization than a lower number of OH groups (Wang *et al.*, 2016).

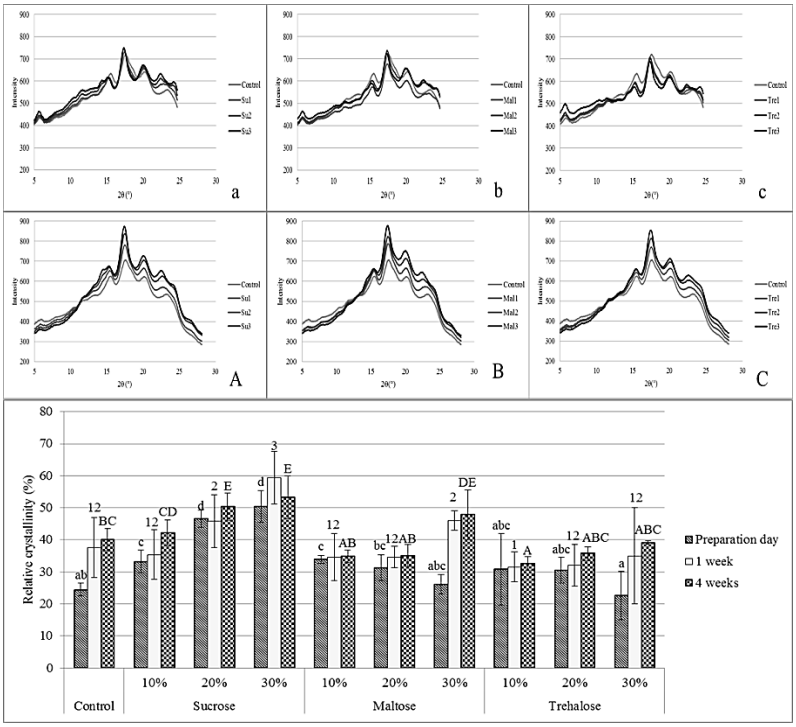


Figure 2: X-ray diffraction pattern and relative crystallinity of rice starch film with and without disaccharide. a, b, and c: x-ray diffraction pattern of rice film with sucrose, maltose, and trehalose, respectively, on the day of preparation. A, B, and C: x-ray diffraction pattern of rice film with sucrose, maltose, and trehalose, respectively, at 4 weeks.

*Different small letters in each column indicates difference on the day of preparation, with significant difference at $p \leq 0.05$.

**Different number letters in each column indicates difference at 1 week, with significant difference at $p \leq 0.05$.

***Different capital letters in each column indicates difference at 4 weeks, with significant difference at $p \leq 0.05$.

3.7 Correlation Test

Pearson bivariate correlation showed high correlation between the concentration of the disaccharides, thickness, transparency, moisture content, water solubility, and mechanical properties as shown in Table 3. The concentration of the disaccharides showed a positive correlation with thickness, moisture content, and water solubility and showed a negative correlation with transparency and breaking stress. From Table 3, the concentration of the disaccharides affected the properties of the rice starch film more than the type of disaccharide. However, suitable conditions for both the type and concentration of disaccharide that interacts with starch in the system are an important factor that affects rice starch film (Chang *et al.*, 2004; Zhang and Han, 2006; Piermaria *et al.*, 2011; Ploypetchara *et al.*, 2015; Sanyang *et al.*, 2015; Shah *et al*., 2015), such as added 10% trehalose showing better properties than the other samples in this experiment.

Table 3: Pearson correlation of properties of rice starch film plasticized by disaccharide.

	Disacc	Conc	thick	trans	mc	soluble	Bstre0	Bstre1	Bstre4	Bsta0	Bsta1	Bsta4	XRD0	XRD1	XRD4
Disacc	1	.000	–.011	.058	–.036	.278	–.129	–.091	–.097	–.246	–.076	.058	–.612*	–.562	–.650*
Conc	…	1	.983**	–.964**	.792**	.926**	–.937**	–.958**	–.961**	–.427	–.928**	–.936**	.385	.462	.428
thick	…	…	1	–.992**	.836**	.893**	–.900**	–.970**	–.971**	–.394	–.960**	–.939**	.420	.378	.353
trans	…	…	…	1	–.886**	–.860**	.848**	.946**	.940**	.306	.941**	.906**	–.488	–.364	–.348
mc	…	…	…	…	1	.757**	–.572	–.730**	–.708**	.122	–.729**	–.620*	.521	.138	.133
soluble	…	…	…	…	…	1	–.885**	–.879**	–.894**	–.364	–.838**	–.792**	.141	.234	.147
Bstre0	…	…	…	…	…	…	1	.929**	.941**	.656*	.887**	.926**	–.145	–.409	–.374
Bstre1	…	…	…	…	…	…	…	1	.992**	.561	.978**	.964**	–.345	–.360	–.316
Bstre4	…	…	…	…	…	…	…	…	1	.568	.970**	.964**	–.308	–.362	–.304
Bsta0	…	…	…	…	…	…	…	…	…	1	.543	.621*	.231	–.246	-–211
Bsta1	…	…	…	…	…	…	…	…	…	…	1	.955**	–.305	–.281	–.280
Bsta4	…	…	…	…	…	…	…	…	…	…	…	1	–.359	–.490	–.498
XRD0	…	…	…	…	…	…	…	…	…	…	…	…	1	.663*	.591*
XRD1	…	…	…	…	…	…	…	…	…	…	…	…	…	1	.924**
XRD4	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1

Disacc = type of disaccharide; Conc = concentration; thick= film thickness; trans = transparency; mc = moisture content; soluble = water solubility; Bstre = breaking stress; Bsta = breaking strain; XRD = relative crystallinity. The number after each treatment indicates storage time (preparation day, 1 week, and 4 weeks). * and ** indicate the correlation is significant at the 0.05 and 0.01 levels (two-tailed), respectively.

4 CONCLUSIONS

The properties of rice starch film incorporated with disaccharide (sucrose, maltose, and trehalose) were investigated for thickness, transparency, moisture content, and water solubility. The mechanical properties and relative crystallization were investigated on the day of preparation and after storage for 1 week and 4 weeks. The results of the experiments showed that film thickness increased with increasing concentration of all three disaccharides, and transparency had a negative relationship with thickness. Moisture content increased with added disaccharide, and water solubility increased with increasing concentration of disaccharide. Water solubility for each concentration increased with an increasing number of equatorial OH groups and hydration number of disaccharide in the order trehalose

> maltose > sucrose. In the mechanical properties of film, the breaking stress of the rice starch film without disaccharide increased, while the breaking strain decreased with increasing storage time. Adding disaccharide decreased the breaking stress of the rice starch film on the day of preparation and after storage. On the day of preparation, adding 10% disaccharide increased the breaking strain, which was caused by the hydrophilic nature of sugar and the number of OH groups. However, in the case of storage, all concentrations of added disaccharide decreased the breaking strain during storage because of the anti-plasticizing effect of the interaction between plasticizer and starch. The intensity and relative crystallinity increased with the increasing concentration of all three disaccharides. Pearson bivariate correlation showed a high correlation between the concentration of the disaccharides, thickness, transparency, moisture content, water solubility, and mechanical properties. Adding disaccharide as a plasticizer affected crystallization and the physical and mechanical properties of the rice starch film based on the type and concentration of disaccharide because of the number of OH groups in the disaccharide structure.

5 ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT).

REFERENCE

- Abu-Jdayil, B., Mohameed, H. A., & Eassa, A. (2004). Rheology of wheat starch–milk–sugar systems: effect of starch concentration, sugar type and concentration, and milk fat content. *Journal of Food Engineering*, *64*(2), 207-212.
- Adjouman, Y.D., NIndjin, C., Tetchi, F.A., Dalcq, A.C., Amani, N.G., & Sindic, M. (2017). Water vapor permeability of edible films based on improved cassava (*Manihot esculenta Crantz*) native starches. *Journal of Food Processing and Technology*, 8, 1–6.
- Apriyana, W., Poeloengasih, C. D., Hernawan, Hayati, S. N., & Pranoto, Y. (2016). Mechanical and microstructural properties of sugar palm *(Arenga pinnata Merr.)* starch film: Effect of aging. *AIP Conference Proceedings*, *1755*(1), 150003.
- Babić, J., Šubarić, D., Milicevic, B., Ačkar, D., Kopjar, M., & Tiban, N. N. (2009). Influence of trehalose, glucose, fructose, and sucrose on gelatinisation and retrogradation of corn and tapioca starches. *Czech Journal of Food Sciences*, *27*(3), 151.
- Buera, P., Schebor, C., & Elizalde, B. (2005). Effects of carbohydrate crystallization on stability of dehydrated foods and ingredient formulations. *Journal of Food Engineering*, *67*(1–2), 157–165.
- Chang, Y.H., Lim, S.T., & Yoo, B., (2004). Dynamic rheology of corn starch-sugar composite. *Journal of Food Engineering*, 64, 521–527.
- Chiu, C., & Solark, D., (2009). Chapter 17: Modification of Starches. In *Starch: Chemistry and Technology*. Third Edition, pp. 640.
- Dias, A. B., Müller, C. M. O., Larotonda, F. D. S., & Laurindo, J. B. (2010). Biodegradable films based on rice starch and rice flour. *Journal of Cereal Science*, *51*(2), 213–219.
- Espitia, P.J.P., Du, W.X., Avena-Bustillos, R.D. J., Soares, N.D.F.F., & McHugh, T.H. (2014). Edible films from pectin: Physical-mechanical and antimicrobial properties-A review. *Food Hydrocolloids* 35, 287–296.
- Famá, L., Rojas, A. M., Goyanes, S., & Gerschenson, L. (2005). Mechanical properties of tapioca-starch edible films containing sorbates. *LWT - Food Science and Technology*, *38*(6), 631–639.
- Farahnaky, A., Saberi, B., & Majzoobi, M. (2013). Effect of glycerol on physical and mechanical properties of wheat starch edible films. *Journal of Texture Studies*, *44*(3), 176–186.
- Galdeano, Melícia Cíntia, Mali, S., Grossmann, M. V. E., Yamashita, F., & García, M. A. (2009). Effects of plasticizers on the properties of oat starch films. *Materials Science and Engineering: C*, *29*(2), 532–538.

- Galdeano, Melicia Cintia, Wilhelm, A. E., Mali, S., & Grossmann, M. V. E. (2013). Influence of thickness on properties of plasticized oat starch films. *Brazilian Archives of Biology and Technology*, *56*(4), 637–644.
- Ghasemlou, M., Aliheidari, N., Fahmi, R., Shojaee-Aliabadi, S., Keshavarz, B., Cran, M.J., & Khaksar, R. (2013). Physical, mechanical and barrier properties of corn starch films incorporated with plant essential oils. *Carbohydrate Polymers*, 98, 1117−1126.
- Hafnimardiyanti, H., & Armin, M.I. (2016). Effect of plasticizer on physical and mechanical characteristics of edible film from mocaf flour. *Der Phamacia Lettre* 8(19), 301−308.
- Ikeda, S., Furuta, C., Fujita, Y., & Gohtani, S. (2014). Effects of D-psicose on gelatinization and retrogradation of rice flour. *Starch/Stärke*, 66, 773−779.
- Jane, J., Shen, L., Wang, L., & Manigat, C.C. (1992). Preparation and properties of small-particle corn starch. *Cereal Chemistry*, 69(3), 280−283.
- Jatmiko, T. H., Poeloengasih, C. D., Prasetyo, D. J., & Rosyida, V. T. (2016). Effect of plasticizer on moisture sorption isotherm of sugar palm (*Arenga Pinnata*) starch film. *AIP Conference Proceedings*, *1711*(1), 080004.
- Kawai, H., Sakurai, M., Inoue, Y., Chûjô, R., & Kobayashi, S. (1992). Hydration of oligosaccharides: Anomalous hydration ability of trehalose. *Cryobiology* 29, 599−606.
- Laohakunjit, N., & Noomhorm, A. (2004). Effect of Plasticizers on Mechanical and Barrier Properties of Rice Starch Film. *Starch - Stärke*, *56*, 348–356.
- Nindjin, C., Beyrer, M., & Amani, G. N. (2015). Effects of sucrose and vegetable oil on properties of native cassava (*Manihot esculenta Crantz*) starch-based edible films. *African Journal of Food, Agriculture, Nutrition and Development*, *15*(2), 9905–9921.
- Piermaria, J., Bosch, A., Pinotti, A., Yantorno, O., García, M., & Abraham, A. (2011). Kefiran films plasticized with sugars and polyols: Water vapor barrier and mechanical properties in relation to their microstructure analyzed by ATR/FT-IR spectroscopy. *Food Hydrocolloids - FOOD HYDROCOLLOID*, *25*, 1261–1269.
- Ploypetchara, T., Suwannaporn, P., Pechyen, A. P. D. C., & Gohtani, S. (2015). Retrogradation of rice flour gel and dough: plasticization effects of some food additives. *Cereal Chemistry Journal*, *92*, 198–203.
- Primo-Martín, C., van Nieuwenhuijzen, N. H., Hamer, R. J., & van Vliet, T. (2007). Crystallinity changes in wheat starch during the bread-making process: Starch crystallinity in the bread crust. *Journal of Cereal Science*, *45*(2), 219–226.
- Razavi, S. M. A., Mohammad Amini, A., & Zahedi, Y. (2015). Characterisation of a new biodegradable edible film based on sage seed gum: Influence of plasticiser type and concentration. *Food Hydrocolloids*, *43*(Supplement C), 290–298.
- Rodríguez, M., Osés, J., Ziani, K., & Maté, J. I. (2006). Combined effect of plasticizers and surfactants on the physical properties of starch based edible films. *Food Research International*, *39*(8), 840–846.
- Russ, N., Zielbauer, B. I., & Vilgis, T. A. (2014). Impact of sucrose and trehalose on different agarose-hydrocolloid systems. *Food Hydrocolloids*, *41*(Supplement C), 44–52.
- Saberi, B., Vuong, Q. V., Chockchaisawasdee, S., Golding, J. B., Scarlett, C. J., & Stathopoulos, C. E. (2016). Mechanical and Physical Properties of Pea Starch Edible Films in the Presence of Glycerol. *Journal of Food Processing and Preservation*, *40*(6), 1339–1351.
- Saberi, B., Chockchaisawasdee, S., Golding, J. B., Scarlett, C. J., & Stathopoulos, C. E. (2017). Physical and mechanical properties of a new edible film made of pea starch and guar gum as affected by glycols, sugars and polyols. *International Journal of Biological Macromolecules*, *104*, 345–359.
- Sanyang, Muhammed L., Sapuan, S. M., Jawaid, M., Ishak, M. R., & Sahari, J. (2015). Effect of plasticizer type and concentration on tensile, thermal and barrier properties of biodegradable films based on sugar palm (*Arenga pinnata*) starch. *Polymers*, *7*(6), 1106–1124.
- Sanyang, M.L., Sapuan, S. M., Jawaid, M., Ishak, M. R., & Sahari, J. (2016). Development and characterization of sugar palm starch and poly(lactic acid) bilayer films. *Carbohydrate Polymers*, *146*, 36–45.

- Shah, U., Gani, A., Ashwar, B.A., Shah, A., Ahmad, M., Gani, A., Wani, I.A., & Masoodi, F.A. (2015). A review of the recent advances in starch as active and nanocomposite packaging films. *Cogent Food & Agriculture*, 1-9.
- Skurty, O., Acevedo, A., Pedreschi, F., Enrione, J., Osorio, F., & Aguilera, J.M. (2010). Food hydrocolloid edible films and coatings. *Nova-Science Publishers*, New York.
- Smits, A. L. M., Kruiskamp, P. H., van Soest, J., & Vliegenthart, J. (2003). The influence of various small plasticisers and malto-oligosaccharides on the retrogradation of (partly) gelatinised starch. *Carbohydrate Polymers 51 (2003) 4, 51*.
- Souza, A. C., Benze, R., Ferrão, E. S., Ditchfield, C., Coelho, A. C. V., & Tadini, C. C. (2012). Cassava starch biodegradable films: Influence of glycerol and clay nanoparticles content on tensile and barrier properties and glass transition temperature. *LWT - Food Science and Technology*, *46*(1), 110–117.
- Tongdeesoontorn, W., Mauer, L. J., Wongruong, S., Sriburi, P., & Rachtanapun, P. (2011). Effect of carboxymethyl cellulose concentration on physical properties of biodegradable cassava starch-based films. *Chemistry Central Journal*, *5*(1), 6.
- Veiga-Santos, P., Oliveira, L. M., Cereda, M. P., & Scamparini, A. R. P. (2007). Sucrose and inverted sugar as plasticizer. Effect on cassava starch–gelatin film mechanical properties, hydrophilicity and water activity. *Food Chemistry*, *103*(2), 255–262.
- Wang, L., Xu, J., Fan, X., Wang, Q., Wang, P., Zhang, Y., &Yu, Y. (2016). Effect of disaccharides of different composition and linkage on corn and waxy corn starch retrogradation. *Food Hydrocolloids*, *61*, 531–536.
- Yoo, D., & Yoo, B. (2005). Rheology of rice starch-sucrose composites. *Starch/Stärke*, 57, 254–261.
- Zhang, X., Li, R., Kang, H., Luo, D., Fan, J., Zhu, W., & Tong, Q. (2017). Effects of low molecular sugars on the retrogradation of tapioca starch gels during storage. *PLoS ONE*, *12*(12).
- Zhang, Y., & Han, J. H. (2006). Mechanical and Thermal Characteristics of Pea Starch Films Plasticized with Monosaccharides and Polyols. *Journal of Food Science*, *71*(2), E109–E118.

Sequential Extraction of Hyaluronic Acid and Collagen from Chicken Eggshell Membrane

Nina Karla Alparce*, Athapol Noomhorm, Anil Kumar Anal

Asian Institute of Technology, Klong Luang, Thailand
*Corresponding Author (nkmalparce@gmail.com)

ABSTRACT

Hyaluronic acid (HA) and collagen are highly valued substances traditionally extracted from animal tissues through complex, arduous processes. But, in this study, both substances were extracted from eggshell membrane through a sequential process composed of simple methods. HA was extracted using two methods: (1) mixing with salt solution and (2) by papain digestion. In the first method, the salt concentration, extraction period, and volume of the extraction solvent were varied. It was found that HA yield initially increases with salt concentration, the highest being 0.88 mg/g sample using 0.5 M NaCl solution, but decreases at higher salt concentration. Meanwhile, in papain digestion, the highest yield, 5.48 mg/g, was obtained from samples incubated at 60 ºC. The HA yield were less at lower incubation temperatures.

Following HA extraction, collagen was extracted from the residual samples with acetic acid solutions of varied pH. The maximum yield was obtained at pH 2. But sample pretreatment by ultrasonication was found to increase yields. The most significant improvement was observed in samples sonicated at 75% amplitude. Meanwhile, the highest collagen yields were obtained from residual samples recovered after papain digestion. Amount of collagen extracted from samples digested at 20 ºC was about 38 mg/g sample but decreased with increasing incubation temperature likely due to collagen denaturation.

KEYWORDS: Hyaluronic Acid, Collagen, Eggshell Membrane, Ultrasonication, Papain Digestion

1 INTRODUCTION

In all sectors, including egg-breaking industries, hatcheries, households, food service industries and other sectors that utilize only the liquid egg (albumen and yolk), the calcified shell and shell membranes are discarded as wastes. These wastes consist approximately 10% of an egg's weight. They are non-edible with little saleable value in their untreated form but is a rich source of many bioactive compounds with high economic value. In 2014, nearly 70 million metric tons of eggs were produced worldwide (Conway, 2015) which translates to around 7 million metric tons of eggshell wastes generated, globally; the bulk of which was disposed in landfills.

The eggshell waste is composed of 94% calcium carbonate, 1% magnesium carbonate, 1% calcium phosphate, and 4% organic matter including the eggshell membrane (ESM) and some constituents embedded in the calcium carbonate layer (Nys and Gautron, 2007; Stadelman, 2000 as cited by Wei *et al*., 2009). The ESM is known to be comprised of at least 70% proteins; 11% polysaccharide including chondroitin sulfate A and B, dermatan sulfate, hyaluronic acid (HA), keratin sulfate and uronic acids; and small amounts of lipids (Devi *et al*., 2012; Nys and Guatron, 2007).

According to various studies (Adams and Franklin, 2006; Long *et al*., 2005; Ohto-Fujita *et al*., 2011), 10% of the proteins that make up the fibrous network of the eggshell membrane (ESM) are collagens of type I, V, and X, which can all be used in various fields. Yu-Hong and Yu-Jie (2009) reported that ESM collagen is comparable to bovine-skin type I collagen in terms of amino acid composition, suggesting that ESM collagen is a potential substitute for mammalian collagen.

Most polysaccharides in ESM belong to a group called glycosaminoglycans (GAGs), which are mostly anionic in nature and made of repeating disaccharide units of hexosamines e.g. fucose, glucosamine, and hexoses (Ruff *et al*., 2000), and uronic acid or galactose (in keratan sulfate). In the

human body, GAGs are important components of connective tissues and are known to play roles in electrolyte control and water retention (Nakano *et al*., 2001). Among all GAGs, hyaluronic acid is the only one that exists as a free polymer - not covalently attached to core proteins to form proteoglycans like all other GAGs i.e. chondroitin and dermatan sulfate are sulfated glycoproteins.

Among the various ESM components, collagen and hyaluronic acid were deemed most valuable. Collagen has a wide variety of applications in cosmetics, functional foods, pharmaceuticals, and biomedical materials. Hyaluronic acid (HA), on the other hand, has been used in eye drops, cosmetics, nutraceuticals, and various other medical or clinical applications.

In most studies about extracting materials from ESM (Long *et al*., 2005; Osouji, 1971; Pi *et al*., 2011; Ponkham *et al*., 2011; Ren *et al*., 2012; Vulganova and Urgeova, 2013; Yu-Hong and Yu-Jie, 2009; Zhao and Chi, 2008), researchers focused on isolating just one compound. However, it seems wasteful to discard ESM after extraction if one or more of the other compounds left can still be recovered in significant amounts. Vlad (2007) patented a method of extracting polypeptides and other valuable materials (i.e. hyaluronic acid, glucosamine, chondroitin, amino acids etc.) from avian ESM. However, the procedures (fermentation and enzymatic digestion) are arduous and expensive.

Moreover, existing and extensively studied methods of collagen and HA extraction have mostly been applied for common sources of collagen and HA other than ESM. Many methods also involve extensive pretreatment that are mainly intended to remove fats from mammalian sources. Thus, this study aimed to sequentially extract HA and collagen from ESM using simple and inexpensive methods, the conditions of which were studied to achieve the highest yield possible. Specifically, the study aimed to (1) determine the effects of NaCl concentration of the extraction medium and their ratio to the sample weight, as well as the extraction period in the HA yield and (2) determine the effects of pH, papain digestion and ultrasonic pretreatment in collagen yield.

2 MATERIALS AND METHODS

2.1 Separation of the Membrane from the Eggshell

Fresh eggshell wastes were obtained from the egg breaking facility of CPF Co., Ltd in Nakhon Nayok, Thailand. The eggshell wastes were soaked in 2% v/v acetic acid solution (100% glacial acetic acid, Merck, Germany) with pH of 2.79 ± 0.02 for 30 minutes at a ratio of 1 kg sample: 4 L solution.The samples were washed 3 times with tap water and then dried in ambient air for 24 hours. The dried sample was transferred in a zip lock bag and kept in the freezer until further processing or immediately ground using a blender with a small mixer goblet (AJ, Crown Tech Advance Co., Ltd, 350 W). Using a fluidized bed dryer (Sherwood Scientific), lighter shell-free ESMs were separated from the shells. The shell-free ESM were kept in a zip lock bag and stored in freezer (~18 ºC) until further use.

2.2 Extraction of ESM Hyaluronic Acid

2.2.1 Extraction with NaCl Solution

Dried ESM particles (5g) were suspended in salt solution of varying concentrations i.e. 0.5 M, 1.0 M, and 1.5 M NaCl. The sample to solution ratio (g: mL) was varied as follows: 1:20, 1:30, and 1:40, Then, the suspension was mixed (Servodyne 50000-25, Cole-Parmer Instrument Co. with mixer head) at 280 rpm at varied durations i.e. 0.5, 1, and 1.5 h. The crude extract solution was decanted from the membranes and centrifuged. The extraction was done 3 times. Two ml of each extract solution was collected and subjected to a modified carbazole assay (Bitter and Muir, 1962) to quantify the HA extracted. After HA extraction, the membranes were set aside for collagen extraction.

2.2.2 Extraction through Papain Digestion

HA was also extracted from ESM by digestion with papain (30000 USP-U/mg) (Merck, Germany) at different temperatures i.e. 20 ºC, 40 ºC, and 60 ºC for 24 hours at a constant enzyme to sample ratio of 20mg: 1g. ESM samples were initially suspended in papain digestion buffer, which consists of 5mM cysteine (L-cysteine from Merck Germany), 5mM disodium EDTA and 100mM

sodium acetate buffer (both from Ajax Finechem Pty Ltd., New Zealand), pH 5.0, and left at room temperature for about an hour. Then the flasks containing the suspension were placed in a shaking water bath then the papain solution was added to the suspension while shaking. The mixture was incubated for 24 hours at the respective temperature levels.

After the 24-hr incubation, the mixture was centrifuged at 7000 rpm for 15 minutes. The supernatant (crude extract solution) was analyzed for HA content while the ESM samples were subjected to acid extraction of collagen (2.3.1).

2.2.3 Statistical Analysis

The data was analyzed using response surface methodology. The individual and combined effects of the variables were discussed based on the response surface plots generated using Design Expert v. 7.0.0 (trial version). The significance of the model and the parameters were determined based on ANOVA for response surface model.

2.3 Extraction of ESM Collagen

Residual ESM samples (after HA extraction) were suspended in 0.1 N NaOH at a sample to alkaline solution ratio of 1:20 (w/v) and was left in the refrigerator for 12 h to remove other proteins and to prevent the effect of endogenous proteases on collagen. The suspension was then centrifuged for 7000 rpm for 10 min, the supernatant was discarded while the precipitates were washed with distilled water twice and then with 0.025M acetic acid solution.

2.3.1 Conventional Acid Extraction

Collagen was extracted from the ESM samples using a procedure adapted from the methods described by Ponkham *et al*., (2011), Bakar *et al*., (2012), Zhao and Chi (2008), Vlad (2007), and Gunasekaran (2003). After washing, the residual ESMs were suspended in 0.5 M glacial acetic acid at a material-to-solvent ratio (g/mL) of 1:15 and stirred for 24 h at room temperature. The pH of the acetic acid solution was varied i.e. 3, 2.5, 2, and 1.5 by adding 1N HCl or 1N NaOH solution prior to the addition of ESM in the acid solution. At certain extraction periods (2, 4, 6, 8, 12, 18, 24 h), 0.8 mL of ESM-free solvent was collected for collagen quantification.

2.3.2 Ultrasonic Pretreatment

In a beaker, residual samples were suspended in acetic acid solution. The suspension was pre-heated at 40ºC for 30 min and was placed in a temperature-controlled steel jacket that serves as a heat exchanger through which cold water passes through. The cooling water temperature was set at 4 ºC. The ultrasonic probe was lowered into the suspension but the end was kept just above the bottom of the beaker.

The amplitude setting of the ultrasonic processor was varied i.e. 100%, 75%, and 25% but the cycle was kept constant at 0.5. The samples were ultrasonicated for 1 hr. Thereafter, all samples were stirred for 24 hours at room temperature as in the conventional acid extraction.

2.3.3 Papain Digestion as Pre-Treatment

Residual ESM samples from the method described in section 2.2.2 were subjected to conventional acid extraction (2.3.1).

2.3.4 HPLC Analysis of Hydroxyproline

HPLC analysis was conducted based on the method described by Ponkham *et al.* (2011) with some modification.

2.3.5 Quantification of Collagen in Crude Extract

The amount of collagen extracted from ESM was estimated using a modified Lowry's method described by Komsa-Penkova *et al*. (1996) and Kiew and Don (2013a).

2.3.6 Purification of Collagen by Salt Precipitation

Following acid extraction, the suspension was centrifuged (7000 rpm x 15 min) and the supernatant was filtered through a fritted disk funnel with Whatman no.1 filter paper. Then, solid ammonium sulfate salt was slowly added to the filtered solution at 4 ºC, up to 50% saturation, and completely dissolved with gentle stirring. Shortly after dissolution of some solid salts, precipitates started to settle down. The resulting suspensions were kept in the refrigerator at least overnight to allow the complete precipitation of collagen and other proteins in the solution. Then, the precipitated proteins were collected through centrifugation at 8000 rpm, washed with distilled water and re-centrifuged. The precipitates were suspended in distilled water, frozen overnight and then freeze-dried in Scanvac CoolSafe 55-4 (300W) at -55 ºC for 24 hours.

2.3.7 UV-Vis Absorbance Measurement

Precipitated collagen from different treatments was dissolved in 0.1M acetic acid, shook for 3 hours at room temperature, and micro-centrifuged at 4 ºC (10,000g x 4 min). The absorbance of the supernatant was measured from 190 nm-600 nm using quartz cuvettes.

2.3.8 SDS-PAGE

The molecular weight of collagen extracted from ESM was determined by SDS-PAGE. Pre-cast gels (Mini-PROTEAN® TGX™, 12%, 15-well comb, 15µL/well), Laemmli sample buffer (4X), 2-mercaptoethanol, running buffer (10X Tris/Glycine/SDS), "Coomassie Brilliant Blue R-250 staining and destaining solutions," and protein standard (Precision Plus Protein™ Dual Xtra Standards) were all purchased from Bio-Rad. The molecular weight of the samples was estimated by comparing the samples' bands to a protein standard ladder.

2.3.9. Statistical Analysis

All treatments were conducted in triplicates. Analysis of variance and Duncan's multiple range test (DMRT) were conducted using the software IBM SPSS Statistics version 21.

3 RESULTS AND DISCUSSION

3.1 HA Extraction

HA was extracted from ESM using NaCl solutions of various concentration, and it was found that increasing the salt concentration increases the HA yield. However, this increase was observed only up to 0.5 M NaCl and the yield decreased thereafter. Further, extracting HA from the same sample, multiple times showed that the yield obtained is highest during the first time and lower in the subsequent extraction (Figure 1).

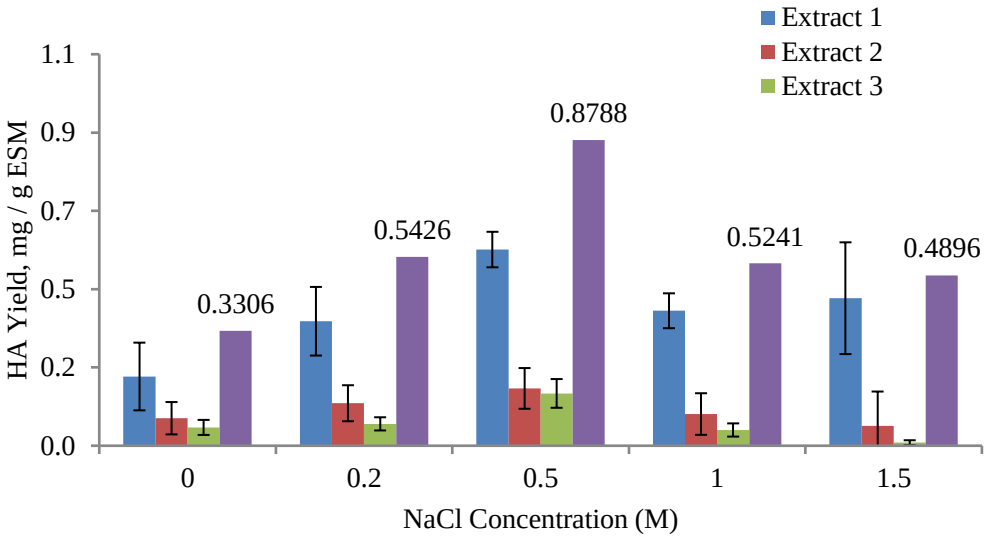
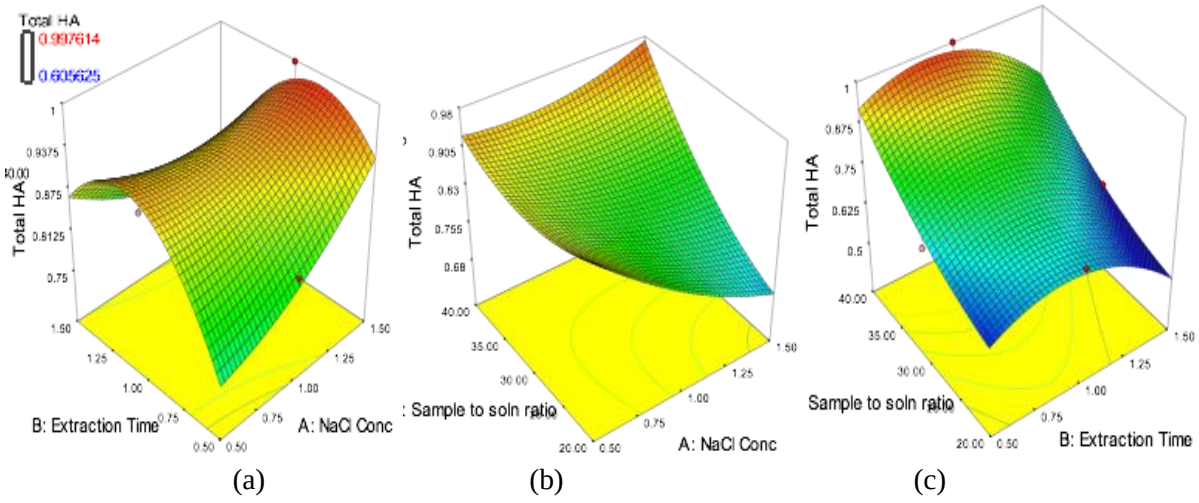


Figure 1: HA (mg/g ESM) extracted using salt solution of varied concentrations.

Analysis by ANOVA and DMRT ($p<0.05$) indicate no significant difference among HA yields at different NaCl concentration. This is likely because there is only a certain amount of HA extractable through this mild method and the yield approaches this amount regardless of the salt concentration. The difference though lies on after how many times the samples should be mixed with extract solution to obtain that said amount. In this case, after the 3rd extraction, the yield from different salt concentrations were already similar to each other and probably close to the total NaCl-extractable HA amount.

To determine the optimum conditions for extracting the maximum amount of HA from ESM using the method employed in this study, a Box and Behnken experimental design was conducted. The response surface plots generated illustrate the individual as well as the combined effects of the independent variables – NaCl concentration, solvent volume to ESM weight ratio, and extraction period – to HA yield.

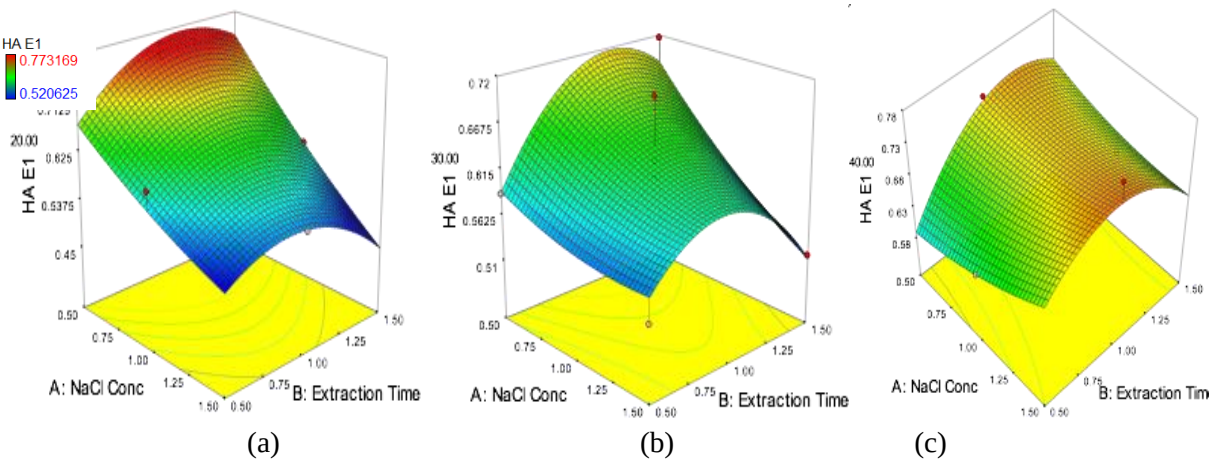
For the total HA yield (Figure 2), the NaCl concentration, solution volume, combined effects of NaCl concentration and extraction period, NaCl concentration and solution volume are the significant parameters in the model.



(a) (b) (c)

Figure 2: Response surface plots for total HA yield at (a) 40 mL NaCl solution/g ESM; (b) 1 h; and (c) 1.5M NaCl Solution.

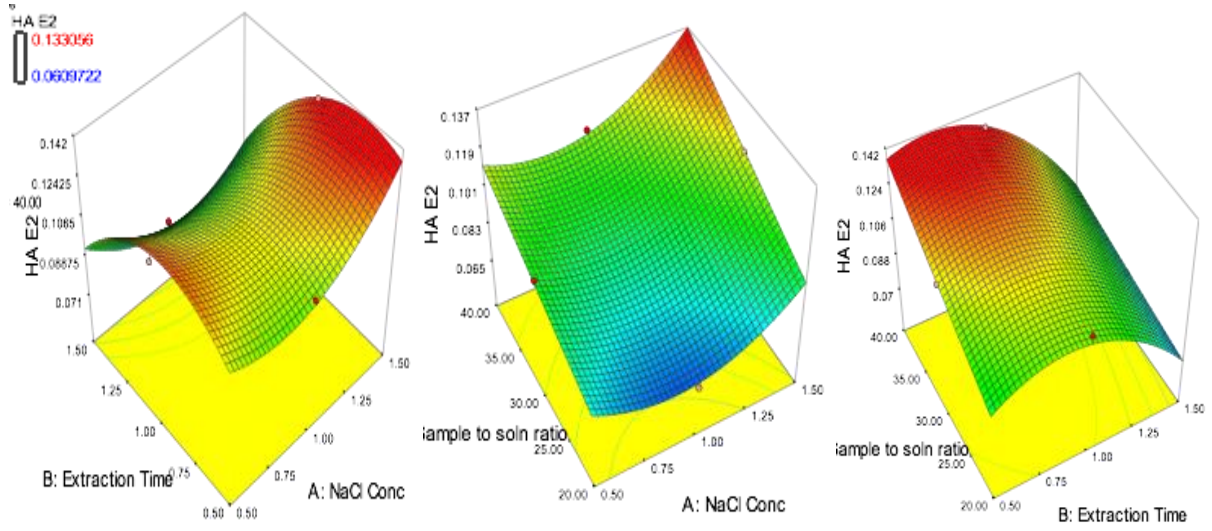
In the analysis of response surface plots for the 1st extraction (Figure 3), it was found that at 20 and 30 mL/g, the HA yield peaks at 0.5 M NaCl solution and at around 1 hour of extraction but decreases at higher salt concentration. However, HA yield can still be increased by increasing the solvent volume i.e. 40 mL/g for the same extraction period.



(a) (b) (c)

Figure 3: Response surface plots for HA yield during the first extraction at different NaCl solution volume: (a) 20 mL/g ESM; (b) 30 mL/g ESM; and (c) 40 mL/g ESM.

In the 2nd extraction (Figure 4), maximum HA yield was obtained in 1.5 M NaCl and at 40 mL of solution/g ESM. This is likely because most of the extractable HA has already been recovered in the first extraction, thus, the recovery of the residual HA in the ESM sample requires greater driving force i.e. higher salt concentration and solvent volume. Moreover, the yield peaked after 30 minutes of extraction and decreased thereafter. This means that with sufficient driving force for diffusion or dissolution, residual HA is extracted faster. Based on the ANOVA for response surface quadratic model, the HA yield is no longer a function of the process parameters which affirms that the yield may depend on the amount of residual HA in the sample and the difficulty of separating these compounds from the ESM matrix.



(a) (b) (c)

Figure 4: Response surface plots for HA yield during the 2nd extraction at (a) 40 mL NaCl solution/g ESM; (b) 0.5 h; and (c) 1.5M NaCl Solution.

During the third extraction, very minimal HA yield was obtained (Figure 5). Based on ANOVA for response surface linear model, among the parameters, only the solution volume is still significant. This means that more solvent is required to extract the residual HA in the ESM samples.

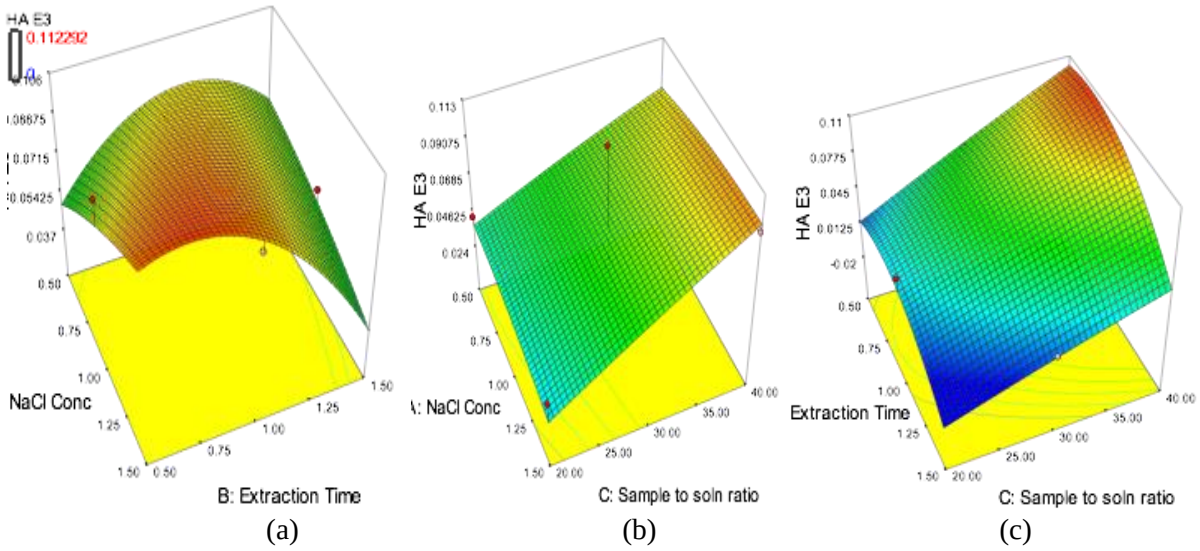


Figure 5. Response surface plots for HA yield during the 3rd extraction at (a) 40 mL NaCl solution/g ESM; (b) 0.5 h; and (c) 1.5M NaCl Solution.

HA was also extracted from ESM by papain digestion with varied temperature (Figure 6). The yield obtained from papain digestion especially at 60 ºC is much higher than that obtained by extraction with NaCl solution.

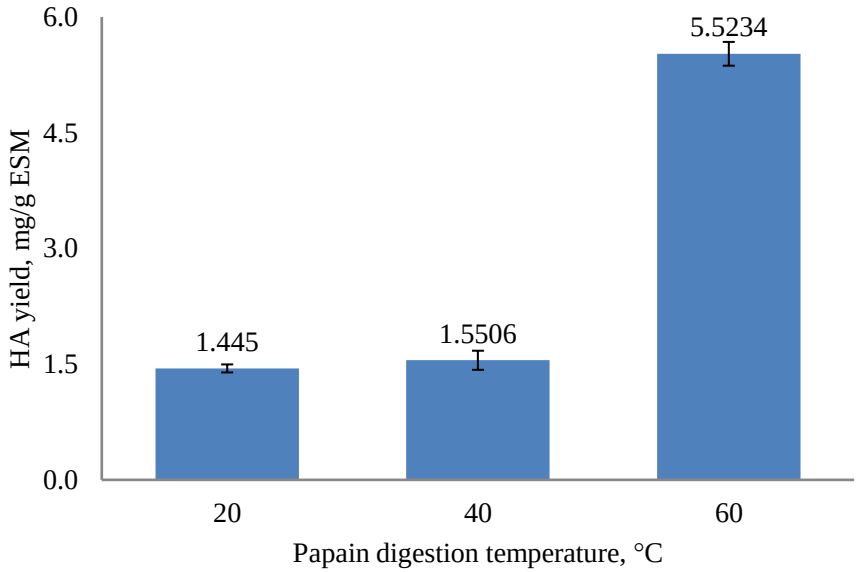


Figure 6: Hyaluronic acid yield from papain digestion of ESM at different temperature

Meanwhile, at lower temperatures of papain digestion, HA yield is lower. This is because the optimum temperature for papain activity is at 60 ºC and at high temperature solubility of other compounds in ESM may have been enhanced consequently loosening the structure and allowing the release of the desired compounds.

3.2 Collagen Extraction

An HPLC chromatogram (figure 7) obtained during the study confirms the presence of collagen in the extract solution. Samples were first hydrolyzed with 6N HCL at 110 ºC to release hydroxyproline (HYP) from the collagen structure. Hydroxproline is a non-essential amino acid that occurs almost exclusively in collagen (Dull and Henneman, 1963).

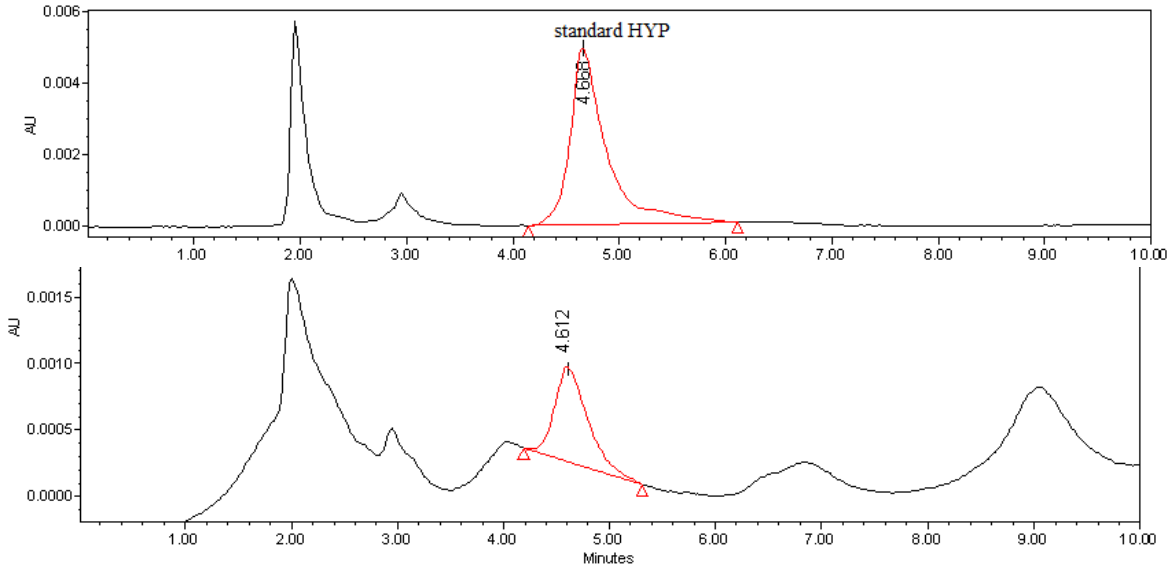


Figure 7: HPLC chromatogram obtained for pure hydroxyproline (top) and for derivatized collagen sample from ESM (bottom).

The retention time of standard hydroxyproline is 4.668 min while that from the sample is 4.612. The very good agreement between the retention times of pure hydroxyproline and that of the sample ascertains that collagen was extracted from ESM and solubilized in the extraction medium. Meanwhile, hydroxyproline was not detected in all pre-treatment washes as well as in the papain digestion buffer after the 24 h incubation.

3.2.1 Effect of pH and Extraction Time on Collagen Yield

The pH of extraction medium was varied from 1.5 to 3. The highest collagen yield was obtained at pH 2 while the lowest was obtained at pH 3 (Figure 8). The yield increased as pH decreased peaking at pH 2. However, a further decrease in pH resulted in lower yield.

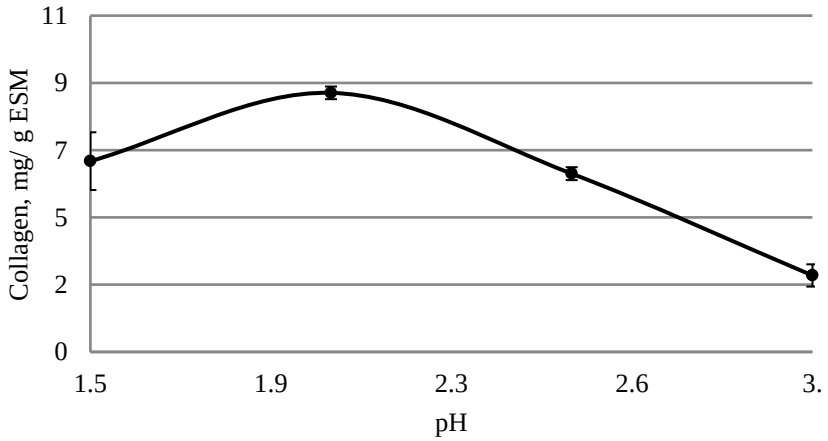


Figure 8: Collagen yield obtained by acid extraction using 0.5M acetic acid of different pH.

Some researchers reported that the isoelectric point (pI) of collagen type I is around pH 8.2 (Uquillas and Akkus, 2012; Zhang *et al*., 2006) while earlier reports put the value at pH 5.5 (Barbani *et al*., 1999 as cited by Zhang *et al.*, 2004). At pH higher or lower than the pI, collagen-solvent interaction increases because it carries a positive or negative charge. And interaction with the solvent will lead to the dissolution of collagen. As the pH of the medium becomes sufficiently far from the pI, collagen solubility is increased and thus extraction yield also increases such as in the case of the present study where yield significantly increased from pH 3 to pH 2.

However, at very low pH, water absorption of collagen may decrease as the amine groups which are positively charged may bind with anions making the electrostatic repulsive forces between the one-nominal charged group weaker (Kiew and Don, 2013b). This results in tightening of the collagen structure consequently reducing the ability to interact with water and thus decreasing solubility in the medium. This explains the decrease in collagen yield at pH 1.5.

During extraction, the change in collagen content of the medium was monitored at 2-hour intervals (Figure 9). Results showed that a marked increase in collagen yield as the extraction progress, particularly at pH 2. However, at the lowest pH (1.5), increase in collagen yield is slower which may be due to the relative difficulty of hydrating the collagen from the samples and thus the reduced solubility of collagen. It is also possible that the collagen was degraded. Similarly, at pH 2.5, increase in yield was also slower which may be explained by the lower amount of H+ in the medium that aid in the hydration of the collagen fibers in the ESM.

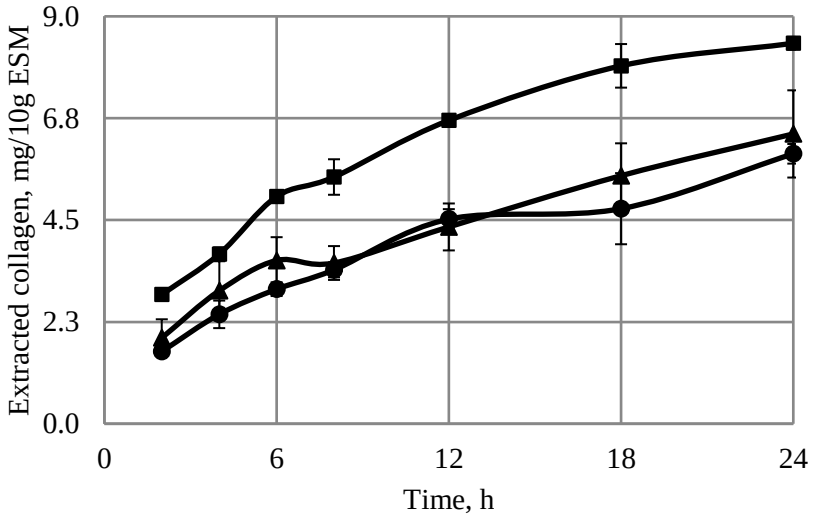


Figure 9: Collagen yield as extraction proceed at different pH (▲, pH 1.5;■ pH 2; ●, pH 2.5)

3.2.3 Effect of Pre-Treatment on Collagen Yield

Ultrasound is utilized in numerous fields and it has also found applications in the food industry. According to Li *et al.,* (2009), "the mechanisms for the thermal, mechanical, and chemical effects of ultrasound are attributed to the rapid formation and collapse of cavitation bubbles." In extraction, the mechanical effect of ultrasound was reported to enhance solvent penetration into the materials and improve mass transfer (Mason *et al.,* 1996). Hydrogen bonds and Van der Waals interactions in the polypeptide chains may also be disrupted, loosening the collagen fibrils and thus enhancing collagen solubility (Li *et al.,* 2009). Hence, in this study, the effect of pre-treating the samples while suspended in the extraction medium at varied amplitudes (constant cycle) for 1 hour on collagen yield was investigated.

The result (Figure 10) indicates that after ultrasonic pretreatment, the collagen yield generally increased as the acid extraction proceeds.

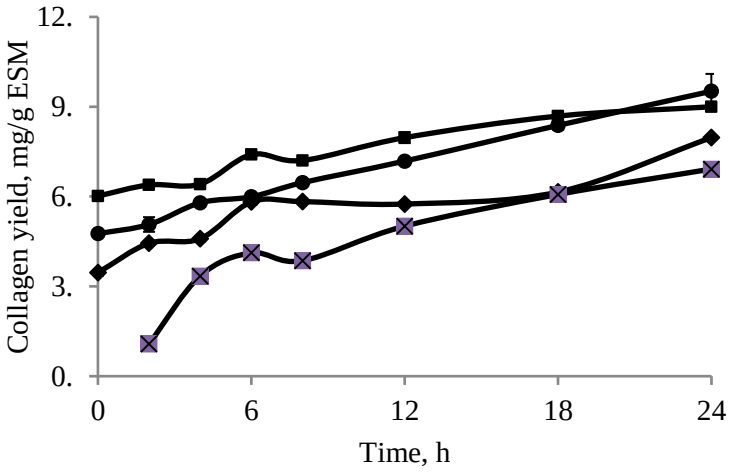


Figure 10: Collagen yield as extraction proceed after ultrasonic pretreatment (■ 100% amplitude; ●, 75% amplitude; ▲, 50% amplitude; x, control)

Increasing the amplitude of vibration will lead to a corresponding increase in intensity and in sonochemical effects (Santos *et al*., 2009). Based on ANOVA, DMRT ($p< 0.05$), the result obtained at 100% amplitude is not significantly different from that of 75% but is significantly higher than at 50%. This implies that higher amplitude is not necessary to achieve desired results and 75% amplitude imposes intensity high enough to achieve cavitation or other sonication effect that improves collagen extraction from ESM. Moreover, Santos *et al.,* (2009) reported that very high amplitudes may "lead to rapid deterioration of the ultrasonic transducer, resulting in liquid agitation instead of cavitation and in poor transmission of the ultrasound through the liquid media." Meanwhile, results obtained at 50% amplitude is not significantly different from that of the control (no ultrasonication) suggesting that at this amplitude, the mechanical, thermal, and chemical effects are not sufficient to improve the collagen extraction from ESM.

3.2.3.2 Effect of Papain Digestion on Collagen Yield

The activity of papain as well as the integrity of the collagen structure is temperature sensitive, hence, papain digestion was conducted at different temperatures i.e. 60, 40, and 20 ºC. The change in collagen yield was monitored at 2-hour intervals. The results (figure 11) indicate that papain digestion was very effective in making collagen in ESM readily soluble or extractable in acetic acid.

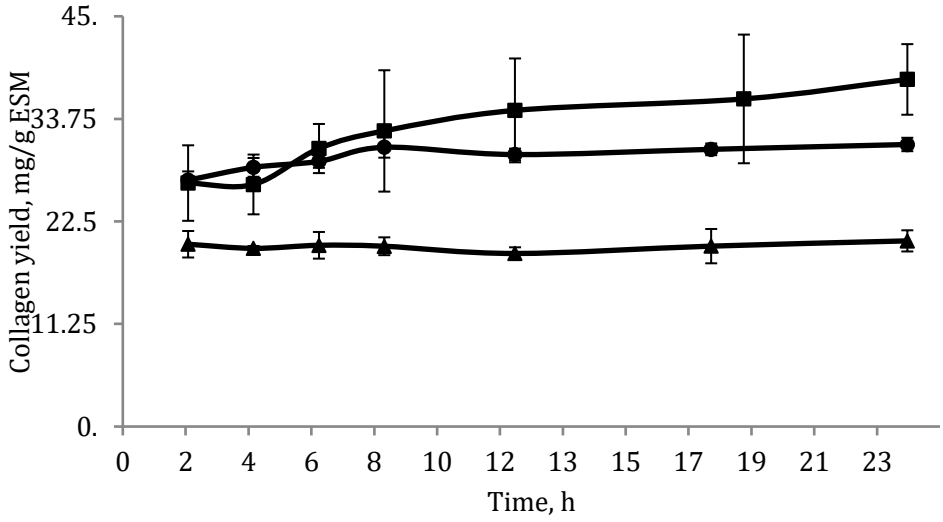


Figure 11: Collagen yield as extraction proceed after papain digestion at 20 ºC (■), 40 ºC (●), and 60 ºC (▲).

Although some protein was certainly extracted or hydrolyzed during papain digestion, salt-precipitated protein from the digestion buffer were characterized by UV Vis absorbance from 190-600 nm, the result of which suggested that they were mostly non-collagenous.

Comparing the collagen yields following the papain digestion pre-treatment at different temperatures, the lowest was obtained at 60 ºC. Although, at this temperature, HA yield was highest, it definitely took a toll on the collagen yield. This is likely because at this temperature, the optimum condition for papain activity, most proteins including collagen were rapidly hydrolyzed during the 24 hour incubation. And upon the suspension in 0.5M acetic acid, all the residual and extractable collagen in the sample was perhaps instantaneously dissolved since the collagen yield after 2 hours remained practically constant over 24 hours.

Meanwhile at 40 ºC, the enzyme activity was lower yielding lower HA amounts but hydrolyzing less collagen. Since there is more residual collagen in the sample, yield during acetic acid extraction was higher than that of samples hydrolyzed at 60 ºC. The collagen yield upon stirring in acetic acid solution started out a bit lower and thereafter increased very gradually that statistical analysis (DMRT, $p<0.05$) detected no significant increase until after 24 hours of stirring.

Papain digestion at 20º C resulted in lower HA yield yet led to higher collagen yields. This is probably because, at 20ºC, the proteins were not denatured and remained intact in the sample but the enzymatic action cleaved sufficient amounts of peptide bonds that allowed acid-soluble collagen to be extracted easily and at higher amounts.

3.2.4 UV/Vis Spectra

Zhang *et al.*, (2011) reported that collagen extracted from carp fish scale has a distinct absorption at 233 nm. Komsa-Penkova *et al.*, (1996) also reported that collagen concentration can be routinely determined by UV absorbance measurement at 230 nm.

In this study, precipitated and freeze-dried collagens were redissolved in dilute acetic acid solution and the UV/Vis spectra of the sample solutions in the wavelength range of 190-600 nm were recorded (Figure 12 and 13). It was found that the absorbance of collagen extracted by acetic acid of different pH and from ultrasonic-pretreated ESM peaked around 230 nm which agrees well with the results obtained in other studies.

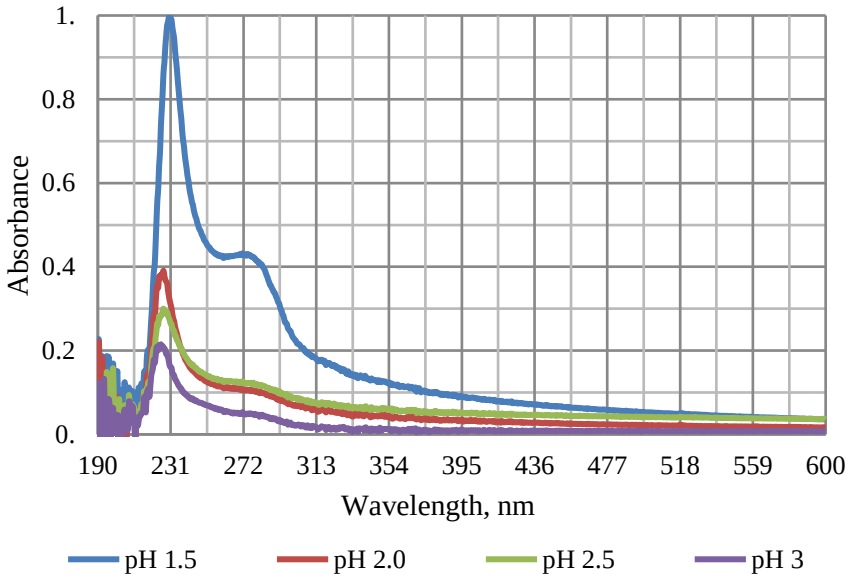


Figure 12: UV-Vis spectra of collagen extracted at different pH

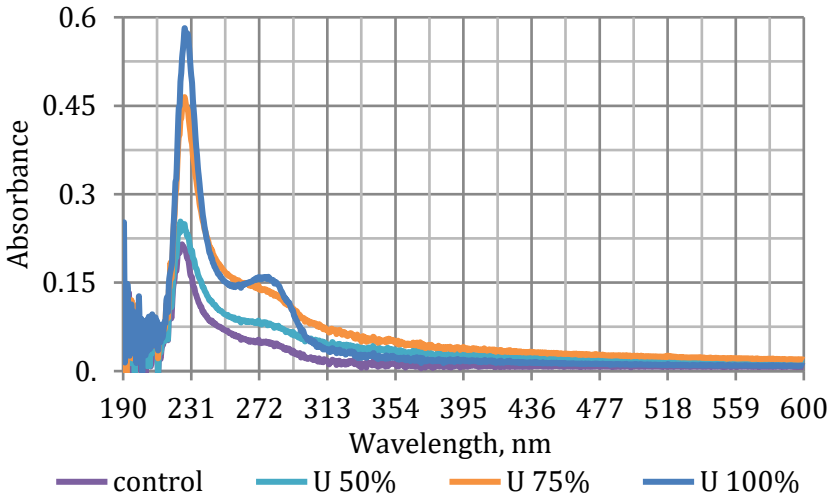


Figure 13: UV-Vis spectra of collagen extracted after ultrasonic pretreatment of ESM at different ultrasonic amplitudes

The shape of the UV-Vis spectra obtained for ESM collagen in this study is also very similar to that of bovine tendon collagen presented by Li *et al*., (2009) (figure 14).

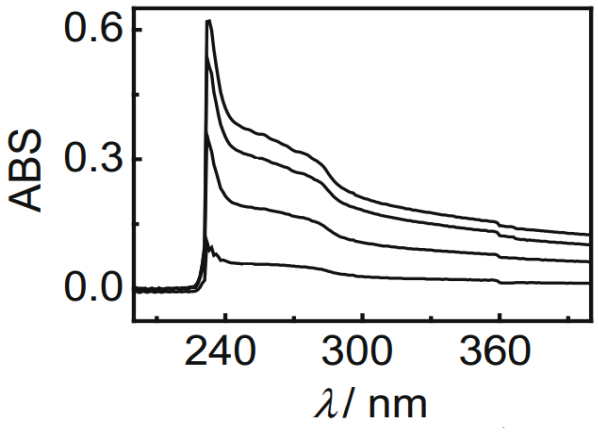


Figure 14: UV-Vis spectra of bovine tendon collagen presented by Li *et al*., (2009)

However, for bovine tendon collagen, the absorbance between 240 and 300 nm are still high indicating the presence of amino acids such as phenylalanine and tyrosine (Li *et al.*, 2009). Whereas, for ESM collagen, absorbance around 270 nm is evidently low. This suggests that the amino acid composition of collagen from different sources may vary. Moreover, the sample obtained at pH 1.5 and from samples ultrasonicated at 100% amplitude showed a small but distinct increase in absorption after 270 nm which may suggest a slightly different amino acid composition than those obtained at higher pH or the presence of non-collagenous proteins.

On the other hand, the shape of the UV-vis spectra recorded for ESM collagen obtained from papain-digested samples (Figure 15) closely resembles the spectra shown in figure 14. This suggests that collagens obtained from papain-digested ESM varies slightly in amino acid content, particularly phenylalanine and tyrosine, from those obtained without pretreatment but at varied pH and those from ultrasonic-pretreated samples. Nonetheless, characteristic absorption was observed at 234 nm for those papain-digested at 20 ºC and 40 ºC and at 230 nm for collagen obtained after papain digestion at 60 ºC.

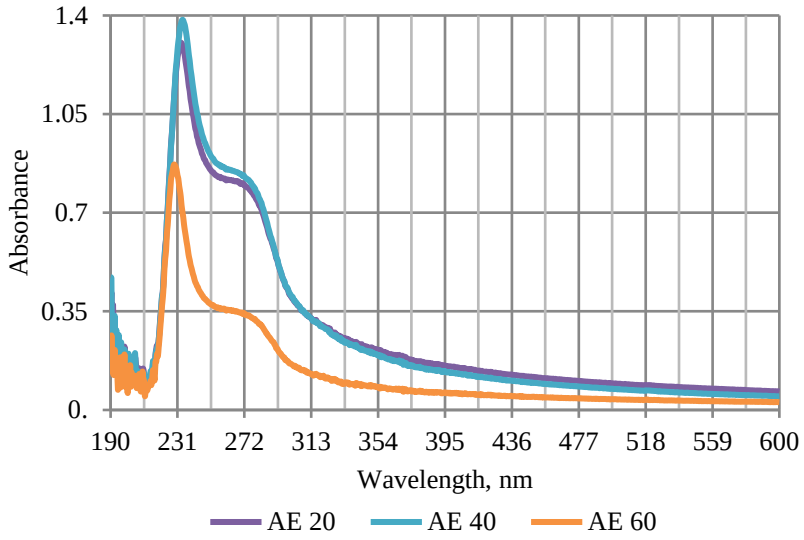


Figure 15: UV-Vis spectra of collagen extracted after papain digestion of ESM at different temperatures

Protein precipitates from the papain buffer was also obtained after HA was isolated. The proteins were freeze-dried and dissolved in dilute acetic acid solution. The UV-vis spectra at 190-600 nm of the resulting solutions showed that the proteins obtained from the papain digest have a characteristic absorbance at about 264 nm which indicates that they are mostly non-collagenous and have considerable amounts of phenylalanine and tyrosine.

3.2.5 SDS-PAGE

In the electrophoresis profile of the precipitated collagen obtained from the different treatments, calf skin type I collagen exhibited clear bands at 250 and 150 kDA corresponding to β chain and α chains, respectively. However, for the collagen extracts in this study, only one distinct band was observed. Nonetheless, SDS-page profile indicates that collagen extracted from ESM by the methods employed in this study has a molecular weight close to that of a standard type I collagen.

4 CONCLUSION

This study has shown that significant amounts of HA and collagen can be sequentially extracted from eggshell membrane using simple and mild methods i.e. (1) extraction with salt solution, and (2) by papain digestion. In the first method, results showed that increasing the salt concentration of the extract solution initially increased the HA yield until 0.5 M NaCl. However, at higher salt concentration, e.g. 1.0 and 1.5 M NaCl, the yields were lower.

HA was also extracted from ESM by varying conditions based on salt concentration, extraction period, and volume of salt solution per unit weight of sample following a Box and Behnken experimental design. Statistical analysis of the data obtained from the experiment indicated that salt concentration and solution volume significant affected HA extraction but extraction time did not. However, the combined effects of salt concentration and extraction time as well as of salt concentration and solution volume have significant impact on HA yield.

Papain digestion resulted in higher HA yields compared to the first method. Digestion at 60 ºC resulted in a yield of 5.483 mg/g ESM, while at 40 ºC, 1.551 mg/g ESM, and at 20 ºC, 1.445 mg/g ESM. Papain is most active at 60 ºC hence resulted in the highest yield while at lower temperatures enzyme activity was lower, yielding less amount of HA.

After HA extraction, samples were subjected to collagen extraction using acetic acid solution (0.5 M) of varied pH levels. It was found that highest collagen yield from acid extraction was obtained at pH 2. At higher pH, yield decreases but at pH lower than 2, yield also decreases. Characterization of precipitated collagen by SDS-PAGE indicated that varying the pH has no detrimental effect on the collagen structure as the molecular weights are still high and close to that of a standard, undenatured

collagen. Meanwhile, the UV-Vis spectra indicated purity of the precipitated collagens as the characteristic absorption was observed at around 230 nm with very low absorption at 270 nm which is characteristic of non-collagenous proteins.

To improve collagen yields, two different pre-treatment method: (1) ultrasonication and (2) papain digestion were studied through single factor experiments. Results showed that ultrasonication improved collagen extraction; the highest was obtained at 75% amplitude. However, characterization by SDS PAGE suggested that collagen obtained from these pretreated samples (except those at 100% amplitude) were slightly denatured as indicated by the slightly lower molecular weights.

The highest amounts of collagen were extracted from papain-digested ESM. It was found that samples digested at 60 ºC had the least residual collagen. Also, these residual collagens were extracted in less than 2 hours of acid extraction. On the other hand, samples incubated at lower temperatures retained their collagen content and hence resulted in higher extraction yield. In this method, the results suggest that there is a trade-off between HA yield and collagen yield. It is therefore necessary to find at which digestion conditions, the yield of each compound will be maximized.

REFERENCES

- Adams, R.G. and Franklin, M.R. (2006). U.S. Patent No. 7,017,277. Washington, DC: U.S. Patent and Trademark Offic
- Bakar, J., Hartina, U., Razali, M., Hashim, D.M., Sazili, Q.A., Kaur, H., Rahman, R.A., Baharin, B.S. (2012). U.S. Patent No. 2012/0114570.
- Bitter, T. and Muir, H.M. (1962). A Modified Uronic Acid Carbazole Reaction. *Analytical Biochemistry*, 4(4): 330-334.
- Conway, A. (2015). World Egg Output grows despite disease impact. http://www.wattagnet.com/articles/25046-world-egg-output-grows-despite-disease-impact/ (accessed 22.04.17)
- Devi, P.S., Banerjee, S., Chowdhury, S.R., Kumar, G.S. (2012). Eggshell membrane: a natural biotemplate to synthesize fluorescent gold nanoparticles. *RSC Advances*, 2: 11578-11585.
- Gunasekaran, S. (2003). U.S. Patent No. 6,548, 077. Washington, DC: U.S. Patent and Trademark Office.
- Kiew, P. L., & Don, M. M. (2013a). Modified Lowry's Method for Acid and Pepsin Soluble Collagen Measurement from Clarias Species Muscles. *Open Access Scientific Reports*, *2*(3), 1-5.
- Kiew, P.L. and Don, M.M. (2013b). The Influence of Acetic Acid Concentration on the Extractability of Collagen from the Skin of Hybrid Clarias sp. and Its Physicochemical Properties: A Preliminary Study. *Focusing on Modern Food Industry*, 2(3): 123-128
- Komsa-Penkova, R., Spirova, R., Bechev, B. (1996). Modification of Lowry's method for collagen concentration measurement. *Journal of Biochemical and Biophysical Methods*, 32: 33-43.
- Li, D., Mu, C., Cai, S., Lin, W. (2009). Ultrasonic irradiation in the enzymatic extraction of collagen. *Ultrasonics Sonochemistry*, 16(5): 605-609.
- Long, F.D. Adams, R.G., DeVore, D.P. 2005. US Patent No. 6,946,551. Washington, DC: U.S. Patent and Trademark Offic
- Mason, T.J., Paniwnyk, L., and Lorimer, J.P. (1996). The uses of ultrasound in food technology. *Ultrasonics Sonochemistry*, 3(3): S253-S260.
- Nakano, T., Ikawa, N.I., Ozimek, L. (2001). Extraction of Glycosaminoglycans from Chicken Eggshell. *Poultry Science*, 80: 681-68
- Nys, Y. and Gautron, J. (2007). Structure and Formation of the Eggshell. In Huopalahti, R., Lopez-Fandiño, R., Anton, M., Schade, R. (Eds.), *Bioactive Egg Compounds* (pp. 99). Springer, Berlin
- Ohto-Fujita, E., Konno, T., Shimizu, M., Ishihara, K., Sugitate, T., Miyake, J., Yoshimura, K., Taniwaki, K., Sakurai, T., Yukio, H., Atomi, Y. (2011). Hydrolyzed eggshell membrane immobilized on phosphorylcholine polymer supplies extracellular matrix environment for human dermal fibroblasts. *Cell Tissue Research*. 345: 177-190.

- Osouji, C.I. (1971). Acid Glycosaminoglycan of Eggshell Membranes. *Biochimica Et Biophysica Acta*, 244: 481-483.
- Pi, Y.-Z., Li, J.-Y., He, L. (2011). Extracting hyaluronic acid from eggshell membrane with double enzyme. *Food Machinery*, 27(4).
- Ponkham, W., Limroongreungrat, K., Sangnark, A. (2011). Extraction of collagen from hen eggshell by using organic acids. *Thai Journal of Agriculural Science*, 44(5): 354-360
- Ren, M., Zhao, S.N., Liu, J.B., Zhang, Y.Y., Sun, Y.B., Yin, J.W. (2012). Enzymatic Extraction of Collagen from Eggshell Membrane. *Food Science*, 33(16): 96-99
- Ruff, K.J., Winkler, A., Jackson, R.W., DeVore, D.P., Ritz, B.W. (2009). Egshell membrane in the treatment of pain and stiffness from Osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clinical Rheumatology*, 28(8): 907-914.
- Santos, H.M., Lodeiro, C., Capelo-Martinez, J.-L. (2009). The Power of Ultrasound. In J.-L. Capelo-Martinez (Ed). *Ultrasound in Chemistry: Analytical Applications*. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
- Uquillas, J.A. and Akkus, O. (2012). Modeling the Electromobility of Type-I Collagen Molecules in the Electrochemical Fabrication of Dense and Aligned Tissue Constructs. *Annals of Biomedical Engineering*, 40: 1641.
- Vlad, V. (2007). U.S. Patent No. 2007/0017447. Washington, DC: U.S. Patent and Trademark Office.
- Vulganova, K. and Urgeova, E. (2013). Extraction of Hyaluronic Acid from eggshell membranes. *Current Opinion in Biotechnology*, Vol. 1 Supplement 1, pages S106.
- Wei, Z., Xu, C., Li, B. (2009). Application of waste eggshell as low-cost solid catalyst for biodiesel production. *Bioresource Technology*, 100: 2883-2885.
- Yu-Hong, Z. and Yu-Jie, C. (2009). Characterization of Collagen from Eggshell Membrane. *Biotechnology* 8(2): 254-258
- Zhang, J., Senger, B., Vautier, D., Picart, C., Schaaf, P., Voegel, J., Lavalle, P. (2004). Natural polyelectrolyte films based on layer-by layer deposition of collagen and hyaluronic acid. *Biomaterial*s, 26 (16): 3353–3361.
- Zhang, Z. K., G. Y. Li, and B. Shi. (2006). Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal- Society of Leather Technologists and Chemists* 90(1):23–28.
- Zhang, F., Wang, A., Li, Z., He, S., Shao, L. (2011). Preparation and Characterisation of Collagen from Freshwater Fish Scales. *Food and Nutrition Sciences*, 2(8):
- Zhao, Y.H. and Chi Y.-J. (2008). Extraction and partial characterization of hyaluronic acid from eggshell membrane. *Science and Technology of Food Industry*, 2008-06

Quality Evaluation of Maltose Syrup Produced from Hydrolysis of Cassava (*Manihot esculenta* Crantz) Starch Using Malted Rice (*Oryza sativa* L.)

Theavy Srey[1]*, Erlinda I. Dizon[2], Katherine Ann C. Israel[2], Leoncio C. Raymundo[2], Wilma A. Hurtada[3], Edralina P. Serrano[4]

[1]Faculty of Agro-Industry, Royal University of Agriculture, Dangkor District, Phnom Penh, Cambodia

[2]Institute of Food Science and Technology, College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines, 4031

[3]Institute of Human Nutrition and Food, College of Human Ecology, University of the Philippines Los Baños, College, Laguna, Philippines, 4031

[4]Postharvest Horticulture Training and Research Center, Crop Science Cluster, College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines, 4031

*Corresponding Author (theavysrey@gmail.com)

ABSTRACT

In maltose syrup processing, Maillard reactions occurred between reducing sugars and protein and resulted in different qualities of syrup. Browning reactions may be desirable or undesirable in some food products. The study evaluated quality of maltose syrup produced from hydrolysis of cassava (Rayong 9 cv.) starch using malted rice (Sen Pidao cv.). Saen Pidao rice seeds were germinated for 10 days in room temperature. α-Amylase activity was determined daily. Significant increased in α-amylase activity was observed on Day 8. Two treatments with different ratios of starch to malted rice (T1: 200g: 32g and T2: 200g: 48g) were prepared and adjusted to various pH levels (4.0, 45, 5.0, 5.5, 6.0 & 6.0). Resulted syrups T2 with pH 5.0 and 5.5 contained high RS and DE. An 8-hour saccharification period with additional overnight storage resulted in decreased pH in extracted juice T1 and T2. Moisture content of syrups ranged from 4.65% – 11.79%. The presence of crude protein in syrups may have contributed to browning reactions. The color of syrups ranged from light brown to dark brown. Browning intensity increased when pH increased. In general, syrups studied were more acceptable than local syrup, especially when malted rice was increased.

KEYWORDS: Maltose Syrup, Malted Rice, Dextrose Equivalent, α-Amylase Activity, Saccharification

1 INTRODUCTION

Maillard reactions occur between reducing sugars and protein or amino acid during thermal processing and storage of foods. Formation of brown color represents one feature of these diverse reactions. Browning must be controlled to avoid undesirable products (i.e., in canned fruits. However, in some cases, browning is highly desirable (i.e. in baking of bread) (Davies *et al.*, 1997). Maltose syrup is a disaccharide with the molecular formula, $C_{12}H_{22}O_{11}$. Maltose is the second important product in biochemical series of glucose chains. By adding of another glucose unit it yields maltotriose and a four-glucose chain is maltostetrose, etc. The next product is dextrin when more units of glucose are added; it is also called maltodextrins and later is starch. Maltose syrups processing is similar to glucose syrups through enzymatic saccharification of liquefied starch (DSSE, 2018).

The pH of the medium can either lower or boost browning in products. Generally, browning is increased with increasing pH value (Wolform & Rooney, 1953). When pH of the medium is between < 3 to > 9, some enzymatic reactions occur complete with the Maillard reaction. A change in pH leads to a change in mechanism of the reaction, therefore, leads to formation of different volatile and colored products (Kroh & Westphal, 1988).

The study generally aimed to evaluate the quality of maltose syrup produced from cassava Rayong 9 cv. and Cambodian indigenous rice Sen Pidao cv. Specifically, the study aimed to: i) identify the best germination time of paddy rice with the highest amylase production; ii) determine the best

processing parameters such as pH, saccharification time, and ratio of substrate (cassava starch) to enzyme (malted rice) for maltose syrup production; iii) evaluate the physicochemical properties [color, pH, % moisture content, total soluble solids (TSS), % reducing sugar, % dextrose equivalent, % crude ash content and % crude protein] of the resulting maltose syrups; and iv) determine the acceptability of maltose syrups from cassava starch.

2 MATERIALS AND METHODS

2.1 Experimental Materials

Paddy rice Sen Pidao cv. produced by the Cambodia Agricultural Research and Development Institute (CARDI), and Cassava Rayong 9 cv. commonly grown by Cambodian farmers were used in the study. The enzyme activity assay was conducted at Postharvest Horticulture Training and Research Center, Crop Science Cluster, College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines and cassava starch and maltose syrup processing were conducted at Faculty of Agro-Industry, Royal University of Agriculture, Cambodia.

2.2 Germination of Paddy Rice

Rice Sen Pidao cv. grains were soaked in sufficient amount of water for 3 days, water was changed twice a day to remove fermented odor. Soaked grains were spread uniformly on plastic tray in slanting position and covered with black plastic bag. Rice grains were sprayed with just enough amount of water twice a day. Amylase enzyme activity in germinated rice grains was checked daily for 10 days and the length of roots and shoots of the representative grains were measured. The day produced highest activity of α-amylase was chosen for processing into maltose syrup.

2.3 Preparation of Malted Rice Powder

Malted rice was dried in a convection oven dryer (kilning) until approximately 20% moisture content, ground and packed in PE bags and stored properly at room temperature for future use.

2.4 Determination of α-Amylase Activity in Malted Rice

2.4.1 Preparation of Acetone Powder

Acetone powder was prepared by homogenizing 1 part fresh rice seedlings tissue with 8 parts frozen acetone in pestle and mortar. The homogenate was vacuum filtered and pellet was washed several times with cold acetone. The residue was air-dried, ground to a fine powder and stored inside the freezer for future use.

2.4.2 Extraction of Enzyme

A 0.2 g acetone powder was homogenized with 10 mL of 0.02 M sodium phosphate buffer (pH 6.9) by using a pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 min at 5 °C and the supernatant was dialyzed overnight against distilled water.

2.4.3 Enzyme Assay

The activity of α-amylase was determined by the loss in the ability to give a blue color with iodine. The assay mixture (7.5 mL) contained 0.5 mL enzyme extract, 1.0 mL soluble starch wherein 1 mg/mL was dissolved in 0.02 M phosphate buffer (pH 6.9), and 5.0 mL 0.005% I_2-0.05% KI solution.

The reaction mixture was incubated for 10 min at 20 °C and reaction was stopped with addition of 1.0 mL 0.1 M HCl. Mixture was read using spectrophotometer at 700 nm. Enzyme activity was calibrated against a standard curve made by plotting known units of purified α-amylase versus 700 nm using method of Serrano (1991). However, due to the unavailability of the purified α-amylase, the

method used was modified as 1 unit α-amylase activity defined as to a cause change of the mixture's absorbance by 0.001/min (1 unit = ΔAbs/min) to the plot of the α-amylase activity at 700 nm at different germination period (day) after which the reaction was stopped by addition of 1.0 mL 0.1 M HCl.

2.5 Preparation of Maltose Syrup

Preparation method of maltose syrup using malted rice was adopted from Quynh and Cecil (1996). The study was done with two different proportions of dried crushed rice seedlings. First treatment was composted of 200g dried cassava starch and 32g dried crushed rice seedlings (T1: 200: 16+16). Second treatment was composted of 200g dried cassava starch and 48g dried crushed rice seedlings (T2: 200: 24+24). Hydrolysis of cassava starch was divided into two batches. The first batch was composed of 200g cassava starch, with 16g (T1) and 24g (T2) of dried crushed rice seedlings separately and 400 ml of distilled water was added into each stainless steel pot and stirred well to avoid lumping of starch. pH was adjusted as follows, not adjusted, pH 4.0, pH 4.5, pH 5.0, pH 5.5 pH 6.0 and pH 6.5. This called reaction step. Another 800 ml of distilled water was prepared separately with different pH levels and boiled later on added into the first batch of starch.

The second batch of crushed rice seedlings composed of 16g (T1) and 24g (T2) and was added into each pot; mixing was continued. After addition of malted rice and water, temperature dropped to approximately 60 °C then it was increased and maintained to approximately 80^{o}C with continuous stirring until the starch was well dissolved. The mixture was cooled down at room temperature to approximately 60 – 62 °C and were left in an oven at 60 °C for 8 hours for saccharification. Mixtures were stand further overnight for a complete process. Starch test was done to check whether the starch was completely hydrolyzed (hydrolysation was complete when there was no blueness after addition of iodine solution). The mixture was taken out of the oven on the following day. The saccharified starch was briefly boiled and filtered using cheese cloth. Extracted juice was boiled until the viscous syrup was obtained. When the syrup was thick enough, the pot was removed from the heat and final product was cooled down. Determination of end point depends on the experience of the processor by observing the way the syrup falls from the stick. This is called gelatinization. In this study, gelatinization process took around 40 minutes. The viscous syrups produced were tested for reducing sugar content, physicochemical properties and acceptability by panel of judges.

2.6 Physico-chemical Analysis

2.6.1 pH

The pH of samples was determined using a calibrated Orion 2 Star Bench-top pH meter.

2.6.2 Total Soluble Solids (TSS) (°Brix)

The resulting maltose syrups were analyzed for TSS by using handheld Atago refractometer.

2.6.3 Color Measurement

Chromameter, CapSure^{TM} was used to read the color of the maltose syrups. In 1976, the CIE officially adopted the modified system as CIELAB with the parameters L^*, a^*, b^*. The L^* indicates lightness (0 to 100) with 0 being black and 100 being white. The coordinate a^* is for red (+) and green (−), and b^* is for yellow (+) and blue (−).

2.6.4 Reducing Sugar Determination

Reducing sugar content of the samples was determined using Dinitrosalicylic Acid (DNS) method (Miller 1959).

The intensity of dark red color was read using UV-Vis spectrophotometer, UVmini-1240 at 570 nm. A series of standards was prepared using glucose (0–500 µg) and a standard graph was plotted. The

reducing sugar of samples was determined from a standard curve prepared from a glucose solution with the following concentrations (ug/mL): 100, 200, 300, and 400.

2.6.5 Dextrose Equivalent

Dextrose equivalent (DE) of the sample was calculated as below:

$$\text{DE} = \frac{(\%\ \text{Reducing Sugars})(100)}{\%\ \text{Dry Substances}} \quad (1)$$

2.6.6 Moisture Content (Oven-drying method)

Moisture content of samples was analyzed using the method adopted by Horwitz (2000). Percentage moisture (w/w) content was calculated using equation below:

$$\%\ MC\ = \frac{(initial\ mass - final\ mass)}{initial\ mass_{sample}} \times 100 \quad (2)$$

2.6.7 Crude Ash

Crude ash content of samples was analyzed using dry ashing method of AOAC (2007) as below:

$$\%\ \text{Ash (dry basis)} = \frac{\text{Weight after ashing} - \text{tare weight of crucible}}{\text{Original sample weight x Dry matter coefficient}}\ \text{x } 100 \quad (3)$$

2.7 Crude Protein

Crude protein content of sample was analyzed by using Kjeldahl method (Horwitz, 2000; WHO, 1973; Greenfield & Southgate, 1992). Crude protein content was calculated using equation (4) and (5) as below:

$$\text{N (g\%)} = \frac{(\text{mL 0.1N HCl sample} - \text{mL 0.1N HCl blank}) \text{x } 0.0014 \text{ x NHCl x } 100}{\text{Weight of sample}} \quad (4)$$

$$\text{Protein (g /100g)} = \%\ \text{Total nitrogen x appropriate nitrogen conversion factor} \quad (5)$$

2.8 Acceptability of the Maltose Syrup

A 9-point Hedonic scale scoring test was employed to test the acceptability of the products through rating on an appropriately defined scale. Twenty eight (28) judges familiar with maltose syrup were randomly chosen to evaluate the products. Score sheet and a glass of water were provided to the judges during evaluation.

2.9 Statistical Analysis

All experiments were done in triplicates. Analysis of Variance and Least Significant Different were performed to find out significant difference between treatments and variables at $p \leq 0.05$.

3 RESULTS

3.1 Changes in Temperature and Relative Humidity (RH) of Paddy Rice at Different Germination Period

Germinating paddy rice seeds temperature ranged from 32.8 °C (Day 4) to 34.4 °C (Day 6); while relative humidity ranged from 47% (Day 5) to 59% (Day 9) with an average of 34 °C and 53%, respectively.

3.2 Shoot and Root Development of Rice Seedlings at Different Germination Period

Day 1 of the germination period (soaked seeds were taken out from water), shoots of paddy rice seedlings had yellow color during the first week and turned to green after plastic bag was removed. Length of shoots and roots of the rice seedlings were significantly difference ($P \leq 0.05$). Roots were slightly longer than shoots. Both roots and shoots started to increase in length on Day 2 (~1 cm) daily. Longest shoots and roots were obtained at Day 10 (13 ± 0.25 and 13.17 ± 0.35 cm), respectively.

3.3 Alpha-amylase Activity of Rice Seedlings During Germination Period

Daily α-amylase activity of paddy rice seedlings was significantly different ($P \leq 0.05$). Amylase activity was increased slowly during the first 3 days and shoot up on Day 8 (366 ± 1.53 unit/ml/min); and it was significantly higher than Days 1 to 7. There was a sharp increase of α-amylase activity on Day 4 - 8 and then slowdown from Day 8 – 10 (Figure 1). Germination period of 8th day was selected for processing of maltose syrup in this study.

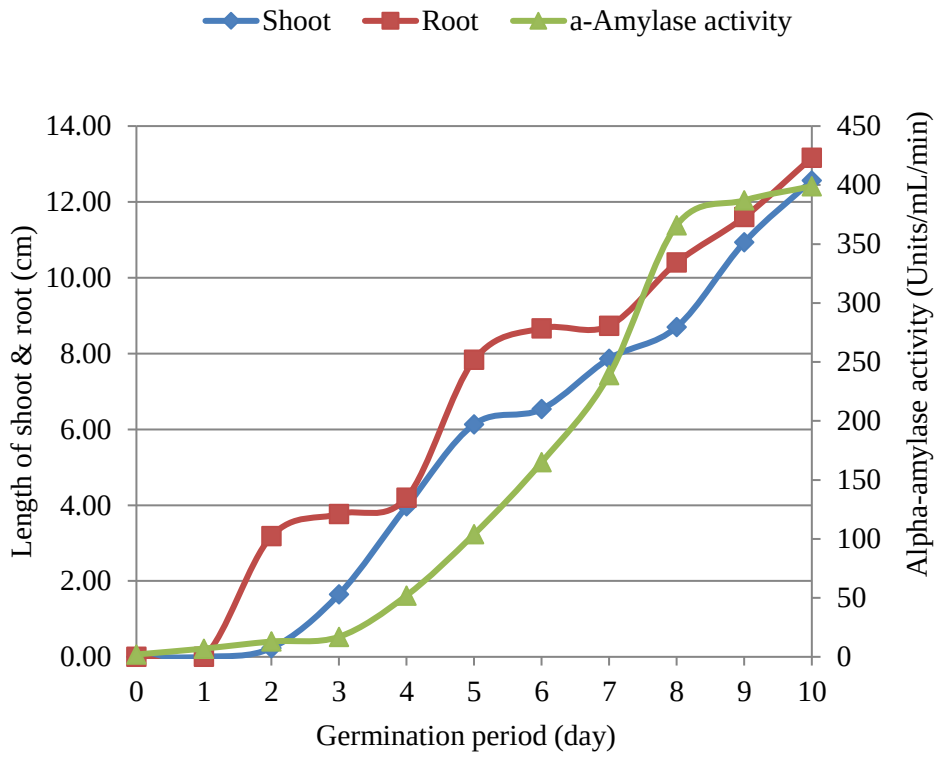


Figure 1: Activity (units/mL/min) of α-amylase in rice seedlings at different germination period (day)

3.3 Reducing Sugar and Dextrose Equivalent of Maltose Syrup

To determine the efficiency of malted rice as enzyme source, percent reducing sugar (%) and dextrose equivalent (%) of maltose syrup from liquefied cassava starch were carried out at different pH levels and results are summarized in Table 1.

Table 1: Reducing sugar (RS %) and dextrose equivalent (DE %) of maltose syrup made from different combination of cassava starch and rice seedlings at different pH levels.

Combination of cassava starch and rice seedlings	pH	RS (%)	DE (%)
T1: Cassava Starch (g) : Rice Seedlings (g) (200 : 16+16)	Unadj. pH (5.3)	91.75 ± 0.50^{b}	83.28 ± 2.04^{bc}
	4.0	82.67 ± 0.38^{d}	79.12 ± 1.88^{cd}
	4.5	68.83 ± 3.19^{f}	64.46 ± 3.01^{f}
	5.0	87.92 ± 5.35^{bc}	81.36 ± 11.29^{bc}
	5.5	87.83 ± 2.89^{bc}	80.38 ± 1.41^{bc}
	6.0	75.58 ± 3.32^{e}	71.29 ± 3.60^{e}
	6.5	87.42 ± 1.38^{bc}	80.21 ± 1.59^{bc}
T2: Cassava Starch (g) : Rice Seedlings (g) (200 : 24+24)	Unadj. pH (5.3)	106.83 ± 0.80^{a}	97.07 ± 0.44^{a}
	4.0	75.75 ± 0.25^{e}	86.61 ± 1.88^{b}
	4.5	66.67 ± 0.63^{f}	64.77 ± 2.85^{f}
	5.0	88.83 ± 3.76^{bc}	85.76 ± 3.80^{bc}
	5.5	89.58 ± 0.14^{bc}	80.71 ± 1.34^{bc}
	6.0	77.33 ± 0.29^{e}	73.16 ± 0.81^{de}
	6.5	86.58 ± 0.14^{c}	80.40 ± 1.54^{bc}

Note: Means in the same column with the same superscripts are not significantly different at $P \leq 0.05$

3.4.1 Reducing Sugar(RS, %)

Treatment without pH adjustment (pH 5.3) had the highest RS for both T1 (91.75 ± 0.50%) and T2 (106.83 ± 0.80%) as compared to other treatments with the same amount of rice seedlings. Samples T1 had significantly higher RS than T2 (Table 1).

3.4.2 Dextrose Equivalent(DE, %)

Dextrose equivalent of syrups of T1 and T2 were significantly difference among the same treatment. DE of T1, sample with unadjusted pH (83.28 ± 2.04%) was significantly higher than others. Likewise, DE of T2, sample with unadjusted pH was significantly higher (97.07 ± 0.44%) from other samples within the same treatment as well; and highest among samples from T1 and T2 (Table 1).

3.5. Chemical Properties of Maltose Syrup

3.5.1 pH

pH values of syrups T1 and T2 were significantly difference ($P \leq 0.01$). pH values of samples with unadjusted pH and pH 5.0 of T1 was 5.27 ± 0.06, higher than other samples. pH values of sample with pH 6.5 of T2 was 5.30 ± 0.00, significantly higher than other samples. pH values of syrups (T1) ranged from 4.57 to 5.27 while pH values of syrups (T2) ranged from 4.53 to 5.30 (Table 2).

3.5.2 Total Soluble Solid (TSS)

Total soluble solid of syrups (T1) and (T2) were significantly different ($P \leq 0.01$). TSS of syrups T1 ranged from 73.00 ± 1.00 to 83.67 ± 0.58 and TSS of syrups T2 ranged from 76.33 ± 0.58 to 83.67 ± 0.58. Syrups with pH 6.5 and with pH 5.0 of T1 and T2 had the same TSS values and higher than other syrups (83.67 ± 0.58 ^{o}Brix) (Table 2).

3.5.3 Moisture Content (MC %)

Moisture content of syrups T1 and T2 were significantly difference ($P \leq 0.01$). MC of syrups T1 ranged from 4.65% to 10.09%. MC of syrup with pH 6.0 was higher than other syrups (11.79%). MC of syrups T2 ranged from 6.58% to 10.14%. MC of syrup with pH 6.5 was significantly higher than other syrups (11.34%) (Table 2).

3.5.6 Crude Ash (%)

Crude ashes of syrups T1 and T2 were significantly difference ($P \leq 0.01$). Crude ashes of syrup T1 and T2 ranged from 0.43 ± 0.03% to 0.64 ± 0.03% and from 0.44 ± 0.03% to 0.66 ± 0.02%, respectively. Crude ash of syrup T1 and T2 with pH 6.0 and 6.5 were not significantly different and higher than other syrups (0.63 ± 0.02% and 0.64 ± 0.03%) and (0.66 ± 0.02% and 0.64 ± 0.05%), respectively (Table 2).

3.5.7 Crude Protein (%)

Crude protein of syrups T1 and T2 were significantly difference ($P \leq 0.01$). Crude protein of syrup T1 and T2 ranged from 0.79 ± 0.11% to 1.82 ± 0.13% and from 0.75 ± 0.07% to 1.52 ± 0.03% (pH 5.0, pH 6.0 and pH 6.5, pH 4.5), respectively. Crude protein of syrup T1 with pH 6.0 was significantly higher than other syrups (Table 2).

Table 2: Chemical properties of maltose syrup from different combinations of cassava starch and malted rice at various pH levels.

Combination of cassava starch and rice seedlings	pH	pH after 8 hr saccharification	Final pH*	TSS (°Brix)*	MC (%)*	Crude ash (%)*	Crude protein (%)*
T1: Cassava Starch (g): Rice Seedlings (g) (200 : 16+16)	Unadj. pH (5.3)	5.6	5.27 ± 0.06^{ab}	76.33 ± 0.58^{e}	8.97 ± 0.47^{d}	0.46 ± 0.03^{c}	1.31 ± 0.08^{cde}
	4.0	4.8	5.03 ± 0.06^{d}	76.67 ± 0.58^{e}	10.09 ± 0.11^{c}	0.46 ± 0.05^{c}	1.43 ± 0.08^{bc}
	4.5	4.9	4.63 ± 0.06^{fg}	80.13 ± 0.23^{c}	4.65 ± 0.56^{g}	0.43 ± 0.03^{c}	1.03 ± 0.05^{f}
	5.0	5.4	5.27 ± 0.06^{ab}	77.33 ± 1.15^{e}	6.20 ± 0.05^{f}	0.45 ± 0.03^{c}	0.79 ± 0.11^{g}
	5.5	5.5	4.70 ± 0.00^{ef}	73.00 ± 1.00^{f}	8.80 ± 0.05^{d}	0.48 ± 0.01^{c}	1.26 ± 0.06^{e}
	6.0	5.9	4.77 ± 0.06^{e}	78.67 ± 0.58^{d}	11.79 ± 0.17^{a}	0.63 ± 0.02^{a}	1.82 ± 0.13^{a}
	6.5	6.2	4.57 ± 0.06^{gh}	83.67 ± 0.58^{a}	7.37 ± 0.22^{e}	0.64 ± 0.03^{a}	1.03 ± 0.05^{f}
T2: Cassava Starch (g): Rice Seedlings (g) (200 : 24+24)	Unadj. pH (5.3)	5.2	4.77 ± 0.06^{e}	80.67 ± 1.15^{bc}	8.77 ± 0.12^{d}	0.44 ± 0.03^{c}	1.45 ± 0.06^{b}
	4.0	4.6	4.53 ± 0.06^{h}	80.47 ± 0.50^{bc}	8.93 ± 0.27^{d}	0.47 ± 0.02^{c}	1.30 ± 0.03^{cde}
	4.5	4.8	4.57 ± 0.06^{gh}	78.67 ± 0.58^{d}	8.89 ± 0.11^{d}	0.55 ± 0.04^{b}	1.52 ± 0.03^{b}
	5.0	5.1	5.20 ± 0.00^{bc}	83.67 ± 0.58^{a}	6.58 ± 0.09^{f}	0.55 ± 0.02^{b}	1.40 ± 0.11^{bcd}
	5.5	5.2	5.13 ± 0.06^{c}	81.67 ± 0.58^{b}	6.58 ± 0.24^{f}	0.58 ± 0.04^{b}	1.28 ± 0.02^{de}
	6.0	5.5	5.03 ± 0.06^{d}	76.33 ± 0.58^{e}	10.14 ± 0.09^{c}	0.66 ± 0.02^{a}	1.05 ± 0.08^{f}
	6.5	5.7	5.30 ± 0.00^{a}	76.33 ± 0.58^{e}	11.34 ± 0.18^{b}	0.64 ± 0.05^{a}	0.75 ± 0.07^{g}

Note: Mean values are ± SD (n=14). Means in the same column followed by the same superscripts are not significantly different ($P \leq 0.05$) by Duncan's Multiple Range Test.
*Significantly different at $P \leq 0.01$; a > b > c > d >….

3.5.8 Color

*Color L**: *L* values of syrups T1 and T2 were significantly difference ($P \leq 0.01$). *L* values of syrups T1 and T2 ranged from 36.33 ± 1.15 to 51.60 ± 1.66 and 30.10 ± 0.52 to 47.07 ± 3.01 (pH 6.5 and pH 4.0), respectively. *L* value of syrup T1 with pH 4.0 was significantly higher than other syrups, which indicated that syrup with pH 4.0 had the lightest color (Table 3). Variation of pH level and starch to malted rice ratio affected to color of syrups. The smaller the pH value, the lighter was the color of the syrup and the lesser the amount of starch to malted rice ratio, the lighter was the color of the syrup (Figure 2).

Table 3: Color values of maltose syrups using Chromameter.

Combination of cassava starch and rice seedlings	pH	Color values		
		*L**	*a**	*b**
T1: Cassava Starch (g) : Rice Seedlings (g) (200 : 16+16)	Unadj. pH (5.3)	36.33 ± 1.15^{ef}	16.33 ± 0.06^{bcd}	25.43 ± 1.75^{d}
	4.0	51.60 ± 1.66^{a}	8.63 ± 0.15^{j}	29.70 ± 0.44^{bc}
	4.5	45.77 ± 2.31^{b}	9.90 ± 0.62^{i}	30.00 ± 0.60^{bc}
	5.0	43.97 ± 0.49^{b}	10.90 ± 0.10^{h}	28.90 ± 0.17^{bc}
	5.5	38.27 ± 2.95^{de}	15.67 ± 0.23^{de}	26.93 ± 3.75^{cd}
	6.0	40.47 ± 2.45^{cd}	15.87 ± 0.67^{cd}	29.33 ± 2.15^{bc}
	6.5	33.13 ± 1.86^{fg}	16.83 ± 0.58^{b}	20.30 ± 2.91^{e}
T2: Cassava Starch (g) : Rice Seedlings (g) (200 : 24+24)	Unadj. pH (5.3)	44.57 ± 1.87^{b}	13.33 ± 0.64^{f}	31.93 ± 0.71^{ab}
	4.0	47.07 ± 3.01^{b}	11.87 ± 0.75^{g}	34.43 ± 1.68^{a}
	4.5	44.60 ± 3.08^{b}	11.90 ± 0.62^{g}	31.47 ± 2.18^{ab}
	5.0	43.70 ± 1.44^{bc}	11.87 ± 0.21^{g}	30.63 ± 1.10^{b}
	5.5	30.90 ± 0.44^{g}	18.50 ± 0.30^{a}	17.70 ± 0.92^{ef}
	6.0	31.23 ± 0.85^{g}	16.77 ± 0.76^{bc}	17.13 ± 1.27^{f}
	6.5	30.10 ± 0.52^{g}	14.90 ± 0.79^{e}	14.70 ± 0.46^{f}

Note: Mean value ± SD (n=14). Means in the same column followed by the same superscripts are not significantly different ($P \leq 0.05$); *Significantly different at $P \leq 0.01$; a > b >…; unadjusted pH was 5.3

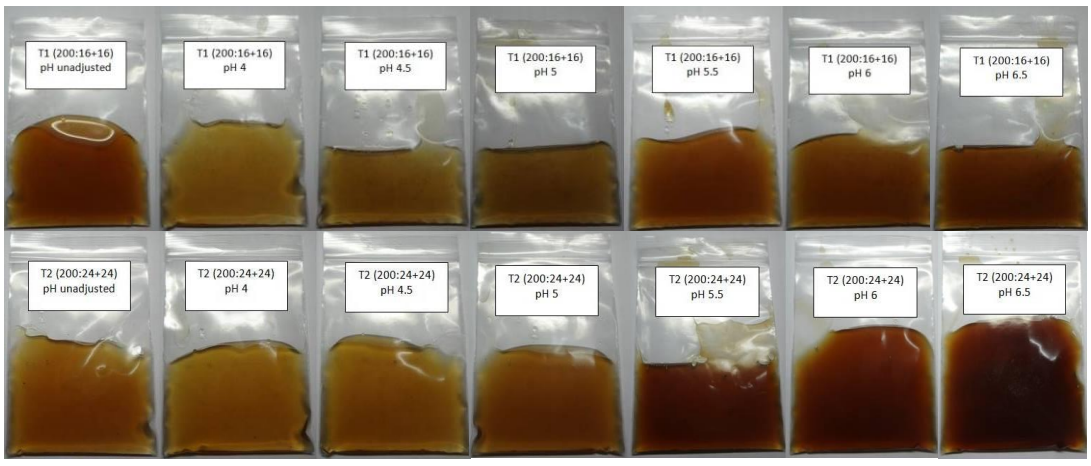


Figure 2: Maltose syrups from hydrolysis of cassava starch at various amount of malted rice adjusted at different pH levels

Color a*: An *a* values of syrups T1 and T2 were significantly difference ($P \leq 0.01$). An *a* value of syrups T1 and T2 were ranged from 8.63 ± 0.15 to 16.83 ± 0.58 and from 11.87 ± 0.21 to 18.50 ± 0.30 (pH 4.0, pH 6.5, pH 5 and pH 5.5), respectively. An *a* values of syrups T1 and T2 from pH 4.0 to 6.5 were increased as pH of samples increased.

Color b*: *b* values of syrups T1 and T2 were significantly difference ($P \leq 0.01$). *b* values of syrups T1 and T2 ranged from 20.30 ± 2.91 to 30.00 ± 0.60 and from 14.70 ± 0.46 to 34.43 ± 1.68 (pH 6.5, pH 4.5, pH 6.5 and pH 4.0), respectively.

3.6 General Acceptability of Maltose Syrup

General acceptability of syrups both T1 and T2 was not significantly difference. All syrups either T1 or T2 were scored from 4.79 ± 2.66 to 6.39 ± 2.04 and from 4.82 ± 2.16 to 6.04 ± 2.15; local syrup was scored at 4.79 ± 2.66 to 4.96 ± 2.40 as compared to T1 and T2, respectively.

Table 4: Mean score of general acceptability of maltose syrup from cassava starch and malted rice.

pH	T1:Cassava Starch (g) : Rice Seedlings (g) (200 : 16+16)	T2:Cassava Starch (g) : Rice Seedlings (g) (200 : 24+24)
Unadj. (pH 5.3)	4.79 ± 2.66	5.21 ± 2.42
pH 4.0	6.39 ± 2.04	5.46 ± 2.32
pH 4.5	5.43 ± 2.33	5.32 ± 2.33
pH 5.0	5.57 ± 2.08	5.43 ± 2.28
pH 5.5	5.00 ± 2.48	6.04 ± 2.15
pH 6.0	5.07 ± 2.29	4.82 ± 2.16
pH 6.5	5.11 ± 2.42	5.82 ± 2.68
Local product	4.79 ± 2.66	4.96 ± 2.40
p-Value	0.2^{ns}	0.6^{ns}

Note: N = 28. Means in the same column followed by the same superscripts are not significantly different ($p \leq 0.05$); ns means not significantly different at ($p \leq 0.05$); range of scores: 1 = Very unacceptable, 9 = Very acceptable

4 DISCUSSION

Paddy rice seeds were germinated for 10 days at temperature and RH ranged from 32.8°C to 34.4°C and from 47% to 59%. Shoots and roots of seedlings were significantly growing from day to day. According Owusu-Mensah *et al.*, (2010), lengths of rice seedlings was increased when increasing malting period; Hammond and Ayernor, (2001), the longer the shoot, the higher was the diastatic power of the rice malt.

α-Amylase activity of rice seedlings was slowly increased during the first 3 days but not significantly difference. However, it was significantly increased on Day 8. Ameko (2013) found out that α-amylase activity reached its peak on Day 4 – 5 and again on Day 10 in germinating seeds. However, in another study by Lenz (1978), amylase activity in maize seeds started 2 - 3 days after absorption of water and reached its peak on Day 6. Lineback and Ponpipom (1977) found out that α-amylase activity increased with increasing of free sugars in all cereals.

Reducing sugar of syrups was increased as the amount of malted rice increased. Syrups with unadjusted pH (5.3) had higher RS than syrups. The result of this study was not far difference from the study of Bruchmann and Fauveau (2010), β-amylase activity was observed around pH 5.2 and generally between 4.8 and 5.5. As studied by Kearsley and Dziedzic (1995), in liquefaction step, α-1,4 linkages are hydrolyzed randomly which led to decrease in the viscosity of the gelatinized starch resulting in increased DE. In this study, when first batch of crushed rice seedlings was added into mixture of starch and water, mixture turned viscous immediately. Liquefying of starch by amylase enzyme was quickly took place after the second batch of crushed rice seedlings was added, especially, with cooked starch. According to Collison (1968), solubility of starch granual in cold water at room temperature was

transformed to more soluble starch at high temperature because lossing of low molecular weight amylose granuals then solution turned viscous and sticky.

Dextrose equivalent of syrups obtained from the present study ranged from 64.46% to 97.07% which are higher than 53, the DE of maltose reported by van der Maarel *et al*. (2002), higher than 40% reported by Ameko (2013), and majority of treatment had DE higher than 50-70% reported by Arasaratnam *et al*., (1998) of syrup obtained from corn starch. According to van der Maarel *et al*., (2002) unhydrolyzed starch paste has a DE of zero, maltrotriose has a DE of 36, maltose has a DE of 53 and a completely hydrolyzed starch (glucose or known as dextrose) has a DE of 100. Therefore, based on the DE values (64.46% – 97.07%) obtained in this study, the cassava starch was almost completely hydrolyzed to glucose. According to van der Maarel *et al*. (2002), the DE of syrup depends on the incubation time and enzyme added. From this study, syrups T1 and T2 with unadjusted pH (5.3) obtained higher DE and RS values than other syrups; and syrups T2 had higher DE than T1.

pH values of syrups T1 were significantly difference. Syrups with pH 5.0 had higher final pH values than other syrups (5.27). pH values of syrups T2 were also significantly higher than other syrups (5.30). Overall, there was no clear trend observed on the effect of initial pH of various treatments on the final pH of syrups. pH values of syrups T1 and T2 ranged from 4.57 to 5.27 and 4.53 to 5.30. These results were comparable to the study of Ameko (2013) which ranged from 4.6 to 5.30 but lower than the values of 5.5 to 6.5 of glucose syrup made from cassava flour and malted rice reported by Dziedzoave *et al*., (2004). According to Marie and Piggott (1991) the pH values of 3.5 - 5.5 of corn syrup were good enough to minimize color and flavor development. Meanwhile, pH values of syrups with unadjusted pH (5.3) was more acceptable for starch hydrolysis by enzyme. Slight decreased of pH values in an 8 hrs saccharified juice and overnight storage may be due to the fermentation that could have taken place before the juice was extracted.

Total soluble solid of syrups T1 and T2 with pH 6.5 and pH 5.0 were the highest (83.67 ^{o}Brix). The TSS of the maltose syrups was higher than 66.6 ^{o}Brix reported by Ameko (2013). Results showed no significantly difference of the TSS of syrups both T1 and T2. However, the lower the MC, the higher was the TSS of the syrup.

Moisture content of all maltose syrups ranged from 4.65 – 11.79% lower than 33.4% reported by Ameko (2013) and 15 - 20% reported by Dziedzoave *et al.,* (2004). There was no substantial effect on MC of syrups as exhibited by unclear trend of the obtained values.

Crude ash of syrups T1 and T2 were not significantly difference. Crude ash of syrups T1 and T2 with pH 6.0 and pH 6.5 were higher than others (0.63% and 0.64%) and (0.66% and 0.64%), respectively. However, crude ash in these syrups were lower than 0.90% crude ash of the syrup obtained from banana starch reported by Bello-Perez *et al.,* (2002), and some treatments contained higher percentage of crude ash than 0.45% obtained from hydrolysis of wet starch with different kinds of maize reported by Ameko (2013); ash content in final product depends on the quality of the starch used. On the other hand, it might be attributed to the NaOH used during pH adjustment, especially, in pH 6.0 and 6.5. According to Kent (2010), acid hydrolysis by mineral acids resulted in a syrup with a high ash content due to neutralization process done with sodium or potassium hydroxide.

There was no clear trend of crude protein in syrups but its values ranged from 0.75% to 1.82%. The small amount of protein content in syrups may be due to the incomplete washing of starch during starch processing. The presence of crude protein could also contribute to the degree of browning in syrups through reaction with the hydrolyzed starch or simple sugars resulting in Maillard browning (Davies *et al*., 1997).

L-value of syrups with pH 4.0 was 51.60 significantly higher than others and the *L*-value decreased when pH of sample was increased. The more acidic the sample, the lighter was the color of the syrup. Raisii and Aroujalianii (2010) reported that browning reaction rate was enhanced with increasing pH value and vice versa. However, there was not trend in T2. Varying amount of malted rice did not affect brightness of syrup.

An *a*-value of syrups T1 was increased as pH increased. The higher the pH value, the higher was the redness of the syrup (dark brown). According to Marie and Piggott (1991), reducing sugar content of syrup such as in glucose, fructose and maltose involved in an occurrence of Maillard reactions. Temperature during gelatinization of syrup also contrbuted to color development in the syrup. The higher the amount of malted rice used, the higher the *a*-value of the syrup. The same to *b*-value of

syrups, *b*-value was lowered when the pH was lowered. Hence, browning reactions can be minized through lowering the pH of the sample.

5 CONCLUSION

Results revealed that cassava starch from Rayong 9 cv. is a potential substrate for maltose syrup production using malted rice as source of amylase enzyme. Significant increased in α-amylase activity was obtained on Day 8 of rice grains germination period and found efficient in the conversion of cassava starch to maltose syrup at this stage. The pH adjustment within the range of 5.0-5.5 and the use of higher amount of malted rice resulted in high reducing sugar (%) and dextrose equivalent in syrup. An 8-hour saccharification period of extracted juice with additional overnight storage resulted in almost complete hydrolysis of the starch.

The presence of crude protein in syrups contributed to the intensity of browning reactions of malted syrups. The color of the maltose syrups ranged from light brown to dark brown; and browning intensity increased with increasing pH and malted rice. In general, syrups studied were more acceptable than local syrup, especially when malted rice was increased.

6 ACKNOWLEDGEMENT

The authors wish to thank IDRC-SEARCA for the scholarship support and the Royal University of Agriculture, Cambodia for an opportunity to finish the research successfully.

REFERENCES

- Ameko, E., Achio, S., Alhassan, S., Gyasi, G., and Sackey, R. (2013). A procedure to determine the germination period for optimum amylase activity in maize malt crude extracts for the artisanal production of maltose syrup from fresh cassava starch. *Innovative Reomaniant Food Biotechnology.* 12: 52-60.
- AOAC International. (2007). Official Methods of Analysis. 18th ed. (2005). Current through revision 2, 2007 (On-line). AOAC International, Gaithersburg, MD. In: Nielsen, S.S. 2010. Food Analysis. 4th ed. ISBN 978-4419-1477-4; Springer New York Dordrecht Heidelberg London.
- Arasaratnam, V., Thayananthan, K., and Balasubramaniam, K. (1998). Isolation and properties of starch from some local cultivars of cassava and taro in Fiji. *The South Pacific Journal of Natural Science* 26(1): 45-48.
- Bello-Perez, L.A., Sanchez-Hernandez, L., Moreno-Damian, E., and Toro-Vazquez, J.F. (2002). Laboratory scale production of maltodextrins and glucose syrup from banana starch. *Acta Cientifica Venezolana*, 53(1): 44-48.
- Bruchmann, A., and Fauveau, C. (2010). Enzyme in potable alcohol and wine production. In Whitehurst, R.J. (Ed). *Enzymes in Food Technology*, 194-209. USA:Wiley-Blackwell.
- Collison, E. (1968). Corn syrup analysis. *Analytical Methods of the Member Companies of the Corn Refiners Association, Inc.* Accepted 5-27-68.
- Davies, C.G.A., Wedzicha, B.L., and Gillard, C. (1997). Kinetics model of the glucose-glysine reaction, *Food Chem.*, vol 60, 323-329.
- Danish Sugar & Sweetener Engineering (DSSE). (2018). Maltose syrups. http://www.dsse.biz/starch-sweetner/maltose-syrup
- Dziedzoave, N.T., Granham, A.J., and Boateng, E.O. (2004). Training manual for the production of glucose syrup. Food Research Institute, Accra, Ghana.
- Hammond, T.K., and Ayernor, G.S. (2001). Characteristic of malted rice for the production of sugar syrup. *Journal of the Ghana Science Association*, 3(2): 91-99.
- Horwitz, W. (2000). Official Method of Analysis of AOAC International. 17th Edition. AOAC International, Maryland, USA. In: Puwastien, P.; Siong, T.E.; Kantasubrata, J.; Craven, G.; Feliciano, R.R.; Judprasong, K. 2011. ASEAN Manual of Food Analysis. 1st ed. Institute of Nutrition, Mahidol University, Thailand.

- Hull, P. (2011). Glucose syrups: Technology and applications. New Jersey: John Wiley & Sons.
- Kearsley, M.W., and Dziedzic, S.Z. (1995). Handbook of starch hydrolysis products and their derivatives. 1st ed. 9-11. New York: Blackie Academic & Professional. Springer.
- Kent, J.A (ed). (2007). Kent and Riegel's Handbook of Industrial Chemistry and Biotechnology. James A. Kent (11th ed), Vol 1; ISBN: 978-0-387-27842-1; Springer Science+Business Media, LLC.
- Kroh, L., and Westphal, G. (1989). Die Reaktion in Lebensmittel. *Chemische Gesellschaft*, 35, 73-80.
- Lenz, D.E. (1978). Hydrolysis of maize endosperm. Master of Science Thesis. McMaster University, Hamilton, Ontario.159 pp.
- Lineback, D.R., and Ponpipom, S. (1977). Effects of germination of wheat, oats, and pearl millet on alpha-amylase activity and starch degradation. *Staerke* 29: 52.
- Marie, S., and Piggott, J.R. (1991). Handbook of sweeteners. New York: Springer Science+Business Media, LLC. 69:664.
- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31(3):426-428.
- Owusu-Mensah, E., Oduro, I., and Sarfo, K.J. (2010). Steeping: A way of improving the malting of rice grain. *Journal of Food Biochemistry*, 35: 80-91.
- Petersen, N. (1975). Edible starches and starch-derived syrup. New Jersey: Noye Data Corporation.
- Quynh, N.K., and Cecil, J. (1996). Sweetness from starch: A manual for making maltose from starch. Food and Agriculture Organization ISBN 10: 9251037809 ISBN 13: 9789251037805.
- Serrano, E.P. (1991). Anaerobiosis and the etiology of internal breakdown in the mango (*Mangifera indica L.* cv Carabao). PhD. Thesis. Postharvest Horticulture Training and Research Center, Library. University of the Philippines Los Banos; Philippines.
- Van Der Marrel, M., Van Der Veen, B., Uitdehaag, J.C.M., Leemhuis, H. and Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology*, 94:137-155.
- World Health Organization (WHO). (1973). Report of a Joint FAO/WHO Ad Hoc Expert Committee on Energy and Protein Requirements, WHO Technical Report Series No. 522. Geneva: WHO. In Puwastien, P. Siong, T.E., Kantasubrata, J., Craven, G., Feliciano, R.R. and Judprasong, K. 2011. ASEAN Manual of Food Analysis (1st Ed.). Thailand: Institute of Nutrition, Mahidol University.
- Wolfrom, M.L., and Rooney, C.S. (1953). *J. Amer. Chem. Soc*., 75, 5435-3436.
- Zainab, A., Modu, S., Falmata, A.S., and Maisaratu, A. (2011). Laboratory scale production of glucose syrup by the enzymatic hydrolysis of starch made from maize, millet and sorghum. *Biokemistri*, Nigerian Society for Experimental Biology 23(1): 1-8.

Extraction and *in vitro* evaluation of antidiabetic efficacy of bioactive extracts from okra (*Abelmoschus esculentus)*

Manisha Anand, Smriti Shrestha, Manisha Singh and Anil Kumar Anal*

Asian Institute of Technology, Pathumthani 12120 Thailand
*Corresponding author: (anilkumar@ait.ac.th; anil.anal@gmail.com)

A**BSTRACT**

Diabetes mellitus is a metabolic disorder of glucose metabolism and it is the most common disorder in developed and developing countries. It has been estimated that up to one-third of pateints suffering from diabetes mellitus use some form of alternative or complementary medicine. Okra plant has received much attention due to its anti-diabetic properties.The present study was designed to identify the bioactive compound (protein,total phenolic content, antioxidant activity) released in the okra extra using distilled water . Moreover, the purpose of the present study was to invetigate the inhibitory activities of α-amylase and α-glucosidase on okra extract. Okra extract exhibited the inhibitory activity against α-amylase (of 11.4 mg/ml) and α-glucosidase (IC_{50} value of 385.44 mg/ml). The inhibitory activity of okra extract may be due to presence of phytochemicals like tannin, flavanoids and saponins.

KEYWORDS: Okra, Diabetes, α-amylase, α-glucosidase, Bioactive compunds,

1 INTRODUCTION

Okra (*Abelmoschus esculentus*) is an annual or perennial herb, commonly known as *lady's finger, gumbo, bhindi, bamia* or *ochro*. It is distributed in tropical, subtropical and warm temperate regions of the world, belonging to the *Malvaceae* family. Okra is valued for its edible green pods, and may serve as alternative sources of protein, fat, fiber and sugar. Okra plays an important role in the human diet by supplying carbohydrates, minerals (potassium, sodium, magnesium and calcium as principle elements with iron, magnese and nickel as trace) and vitamins (Moyin-jesu., 2007 and Arapitsas, 2008). Okra seeds could serve as alternate rich sources of sugar, protein, dietary fibers, fat and phenolic compounds. Okra is a good source of flavonoid compounds, which have antioxidant activities like quercetin 3-O-gentiobioside and isoquercitrin. Epidemiological studies have suggested that the consumption of foods rich in flavonoid compounds reduce the risk of cardiovascular diseases, diabetes, obesity, certain forms of cancer, stroke and hyperlipidemia, suggesting that the consumption of okra may be of benefit for metabolic diseases (Oyelade *et al.*, 2003).

Diabetes mellitus is considered to be in top five prime diseases which causes death in the world. It is normally a syndrome of disordered metabolism or genetic which is usually environmental or hereditary causes. Resulting increase in blood sugar level. It is rapidly increasing and becoming the third most fatal disease amongst mankind, found all over the world, affecting 16 million people in United States and 200 million people worldwide. Normally, general medicines are used to cure but some alternative medicines are also used like herbs and diiferent dietary supplements as alternative to mainstream western medical treatment. A recent study assessed about 30% of patients with this disease use alternative or complementary medicine. Many traditional medicines are made up of plants, organic matter and minerals. For medicinal purpose, 21000 plants are reported by World Health Organization (WHO) which are mainly used for medicinal purposes around the globe. Amongst them, about 150 species are used on a large commercial scale.

Most research focused that okra can be used as medicinal plant and is anti-diabetic plant. Many scientists are more interested in study of its bioactive compounds and their effects on the human body. A number of study reported that the extract of okra contains numerous bioactive compounds and they have hypoglycemic action in both diabetic human and animals. Recently one study reported that the powder of okra play antihyperlipidemic and antidiabetic characters in STZ induced diabetic rats. Recently, researchers found that okra polysaccharides reduced the weight of body, improved glucose

level and glucose tolerance, decreased serum total cholesterol (TC) level in the high fat diet served mice. In addition, okra successfully decreases tumor necrosis factor α levels in T3-L1 adipocytes. It indicates that the okra plays a major role in lipid metabolism and regulating the glucose level. In the present study, it is concluded that okra has major phenolic compound and antioxidant activity.

2 MATERIALS AND METHODS

2.1 Materials

Okra samples were bought from a local Taladthai, Pathumthani. Size of okra was 2-3 inches long, green in color, same maturity (harvested after two month of planting). All chemicals were of analytical grade.

2.2 Preparation of Plant Extracts

The okra pods were cleaned efficiently with distilled water, dried in hot air oven, and finally, ground into fine powder. The okra powder were used to prepare okra extract following the method as described by Adwan *et a*l., (2010) with slight modification. 30 g okra powder in in 250 ml of ethanol (80%, v/v) was incubated on shaking incubator (Gallenkamp, UK) for 48 hours at 200 rpm. The extracts were filtered by whatman filter paper 1,concentrated by means of rotary evaporator (Büchi rotavapor R-144, Switzerland) and then lyophilized in a freeze dryer (Scanvac Cool Safe 55-4, Denmark). The freeze dried extract powder was stored in a refrigerator at 4 °C.

2.3 Pretreatment of Okra by Soaking

Aqueous okra sample was prepared by soaking sample 25 g in 250 ml in distilled water over night (12 hr) at room temperature. The residue was separated and the aqueous sample was used for determining the bioactive compounds.

2.4 Quantitative Analysis of Bioactive Extracts

2.4.1 Dtermination of Total Phenolic Content (TPC)

The total phenolic content present in the sample was determined by Folin-Ciocalteau method (Kukric et al., 2012). 1.0 ml of the diluted extract and 1.0 ml of Folin-Ciocalteau reagent (1 N) was dissolved in the water at ratio of 1:1 followed by addition of 0.8 ml of 7.5% Na_2CO_3. After incubating the mixture (in dark) for 1 h at the room temperature, the absorbance was measured at 765 nm on a spectrophotometer. The Gallic acid of different concentrations was used to prepare a standard Gallic acid curve.

2.4.2 Determination of Antioxidant Activity

For the estimation of scavenging activity of antioxidant against DPPH radical, DPPH working solution (0.04 g DPPH/L of ethanol) was prepared following Marinova et al., (2011). 1.5 ml of DPPH working solutions was allowed to react with 1.5 ml of the diluted sample. Then, the mixture was incubated for 30 min at room temperature (in dark). DPPH ethanol solution without sample was used as control and 95% ethanol was used as the blank. The decrease in the absorbance after addition of the sample was measured at 517 nm. By the help of followed relation radical scavenging activity was calculated:

$$\text{DPPH scavenging activity (\%)} = \left\lfloor 1 - \left(\frac{\text{test sample absorbance}}{\text{control sample absorbance}} \right) \right\rfloor \times 100(\%) \quad (1)$$

2.4.3 Determination of Protein Content

Protein content was determined following Bradford method. Bradford dye reagent was diluted with water in the ratio of 1:4. 100 µl of sample was added into 5 ml of Bradford dye solution and the mixture was incubated at the room temperature for 15 min. The absorbance was measured by spectrophotometer at 595 nm wavelength. The standard curve was made by using Bovine serum albumin (BSA) at the different concentrations of water.

2.5 Anti-diabetic activity of okra extract in inhibition of α-amylase

The anti-diabetic activity of okra extractin inhibition of α-amylase was determined by using a slightly modified assay described by Kazeem et al., (2013). 250 μL of okra extract (1.25-10 mg/ml) was taken in a test tube and then same amount of sodium phosphate buffer containing α-amylase solution of 0.5 mg/ml was added. This solution was incubated for 10 min at 25 °C. 250 μL of starch (1%) solution in 0.02 M sodium phosphate buffer of pH 6.9 was added at certain time intervals and then further incubated at 25 °C for 10 min. By adding 500 μL di-nitro-salicylic acid (DNS) reagent the reaction was terminated. Then the tubes were kept in hot water bath for incubation at boiling temperature and then cooled to room temperature. Lastly 5 ml of distilled water was added into the reaction mixture and absorbance was measured with using spectrophotometer at 540 nm. A control was prepared by the help of same procedure by replacing the extract with distilled water. The α-amylase activity was calculated by using given equation:

$$\text{α-amylase inhibitory activity} = \frac{((Ac+) - (Ac-) - (As - Ab))}{(Ac+) - (Ac-)} \times 100 \quad (2)$$

Where; Ac+ = the absorbance of 100% enzyme activity (only solvent with enzyme),
Ac- = the absorbance 0% enzyme activity (only solvent without enzyme),
As = A test sample (with enzyme) and
Ab = A blank (a test sample without enzyme)

2.7 Anti-diabetic activity of okra extract in inhibition of α-Glucosidase

The α-glucosidase inhibition assay was done as described by Kazeem et al., (2013) with slight modifications. Substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer (pH 6.9). 100 μL of α-glucosidase 1.0 U/ml with 50 μL of various concentration (ethanol, acetone and water) of okra extracts were preincubated for 10 min. After that 50 μL of 3.0 mM (pNPG) as a substrate was dissolved in 20 mM phosphate buffer with pH 6.9 and then reaction was started. This mixture was incubated for 20 min at 37 °C and 2 ml of 0.1 M Na_2CO_3. The abosorbance was measured at 405 nm by using spectrophotometer and. the value of anti-diabetic activity of extract in inhibition of α-glucosidase was calculated using following equation:

$$\text{Inhibition (\%)} = \frac{\text{Abs 410 (control/Acarbose)} - \text{Abs 410 (extract)}}{\text{Abs 410 (control Acarbose)}} \times 100 \quad (3)$$

From the graph plotted between percent inhibition and log inhibitor concentration the IC_{50} values was calculated and determined by non-linear regression analysis on the basis of mean inhibitory values. Acarbose was used as the reference α-glucosidase inhibitor. All tests were performed three times.

3 RESULTS AND DISCUSSION

3.1 Preliminary Screening of Phytochemicals

The qualitative screening of phytochemicals in the okra revealed the presence of phenols, tannins, alkaloids, terpenoids, flavonoids, glycosides but steroids and saponins were absent. The study of presence of phytochemicals helps to understand the nutritional and pharmacological significance of

the okra. In addition to these compounds, okra pods are rich in biologically important compounds like phenolic components, proteins, amino acids chlorophyll, minerals, fatty acids, minerals, flavanoids, lecithin, vitamins and carotenoids (Kukric, et al, 2012). The biological activity of okra can probably be attributed to high composition of phenolic compounds 83.22 ± 0.5 (pod), 21.5 ± 2.23 (skin) , 13.2 ± 1.02 (seed) (mg/g GAE), antioxidant activity of 50.93 ±1.03 % and protein of 28.5±2.23 (pod), 21.5 ± 2.23 (skin), and13.2 ± 1.02 (seed) (mg/g DW).

3.2 Effect of Pre-Treatment on the Total Phenolic Content

The TPC of soaked sample (seed) was 1% after 12 h incubation which was found to be increased after giving heat treatment at 40 °C for 15 min to 2%. Similarly in peel and pod after soaking for 12 h the TPC were 6 % and 15% , that was observed to increase up to 9% and 20% respectively. However, no significant difference was observed during 0-10 hr of soaking. On the other hand, TPC value was found to decrease withincrease in temperature and time (up to 60 °C for 60 min) due to high temperature and longer time duration.

3.3 Effect of Pre-Treatment on the Protein Content

The protein of soaked okra in seed, peel and pod were 17%, 19% and 23% (12 h of soaking). After giving heat treatment at 60 °C for 30 min protein value increased up to 27.2% and 36.1% for peel and pod respectively, whereas in seed at 60 °C for 15 min the value of protein was 30.2%. Similar report was given by Adelakun et al., 2009 in which the value of protein of okra flour after 18 hr of soaking was 44.03%. About 5 % decrease was observed during 36 hr of soaking of okra.

3.4 Effect of Pre-Treatment on the Antioxidant Activity

After 12 h of soaking, the DPPH radical scavenging activity of okra was increased from 50.93% in the raw sample to 93.2% in the soaked sample. Further, scavenging activity increased by 39% by giving heat treatment at (40 °C for 30 min). However, withincrease in time and temperature, the value of DPPH decreased. Some researcher found that thermal treatment decreases the antioxidant activity content in most of the fruit and vegetables (Adelakun et al., 2009).

3.5 *In-vitro* Evaluation of Anti-Diabetic Activity of Bioactive Extracts from Okra

3.5.1 Anti-Diabetic Activity of Extraction in Inhibition of α-amylase

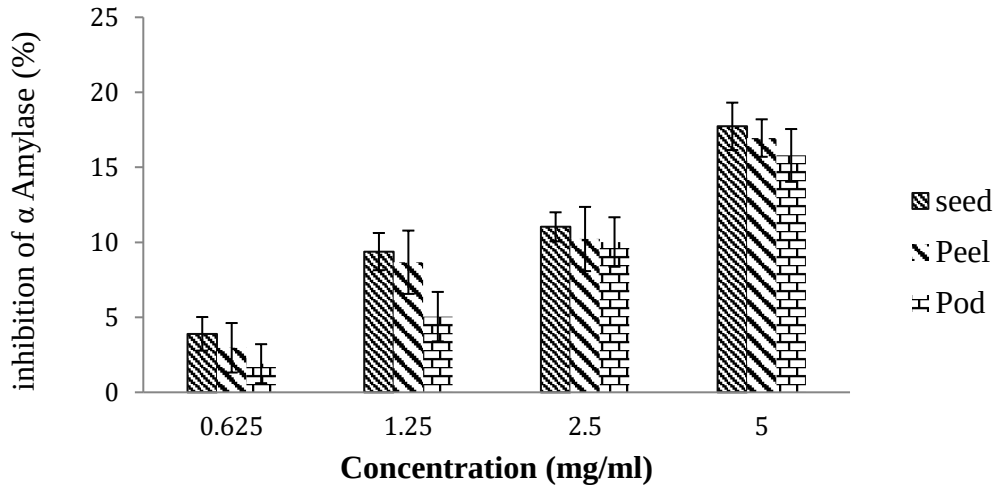


Figure 1: Inhibitory potency of okra extract (Seed, Peel and Pod) against α-amylase

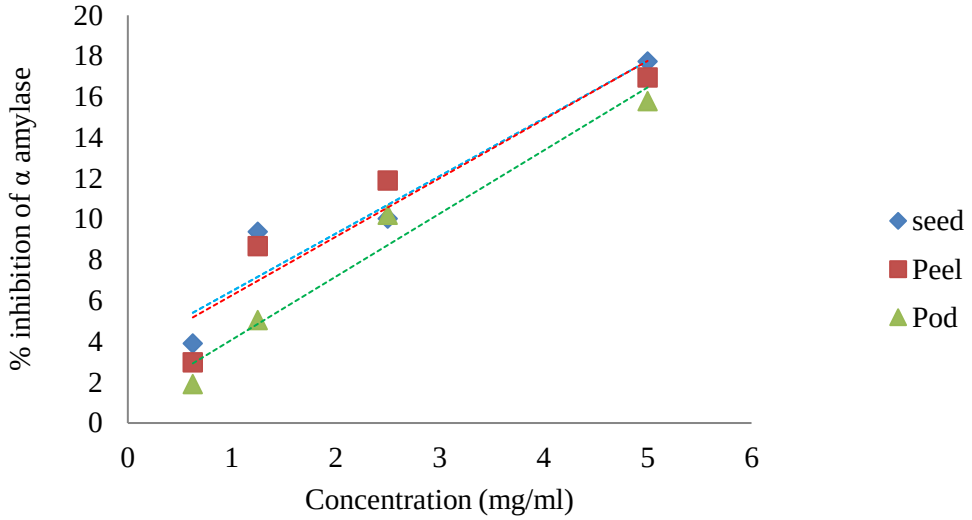


Figure 2: Inhibitory effect of percentage α-amylase (IC_{50} value)

Potential of α-amylase inhibition of the okra extracts was determined as shown in Figure 1 and2. The inhibitory effect of α-amylase by the all extracts at higher concentrations (5 mg/ml) and at lower concentration (0.625 mg/ml) showed slight difference between each other while at higher concentrations (5 mg/ml) potential of aqueous extract was significantly different if compared to another extracts. Above graph showed the α –amylase activity of seed at 5 mg/ml was higherst (17.78 mg/ml) than peel and pod okra extracts. The lowest α amylase was found at 0.625 mg/ml concentration in pod okra which was 1.09 mg/ml. That extract contained the most effective α-amylase inhibitor with IC_{50} value of 11.4 mg/ml. The mode of α-amylase activity of okra aqueous was determined by graph which showed that the extract displayed a near competitive inhibition of enzyme activity.

3.5.2 Anti-diabetic Activity of Extraction in Inhibition of α-Glucosidase

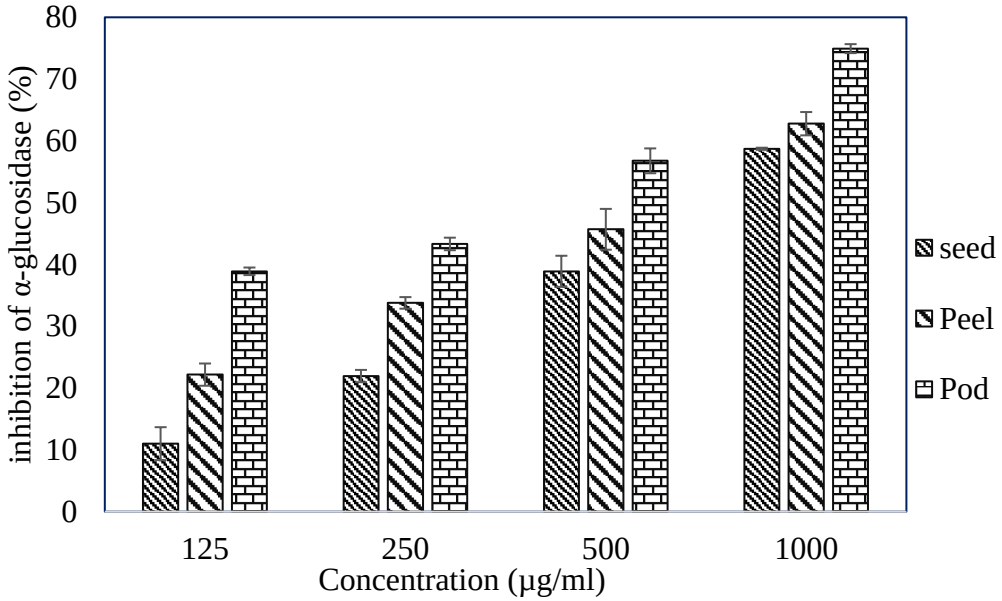


Figure 3: Inhibitory Potency of Okra Extract (Seed, Peel and Pod) against α-glucosidase

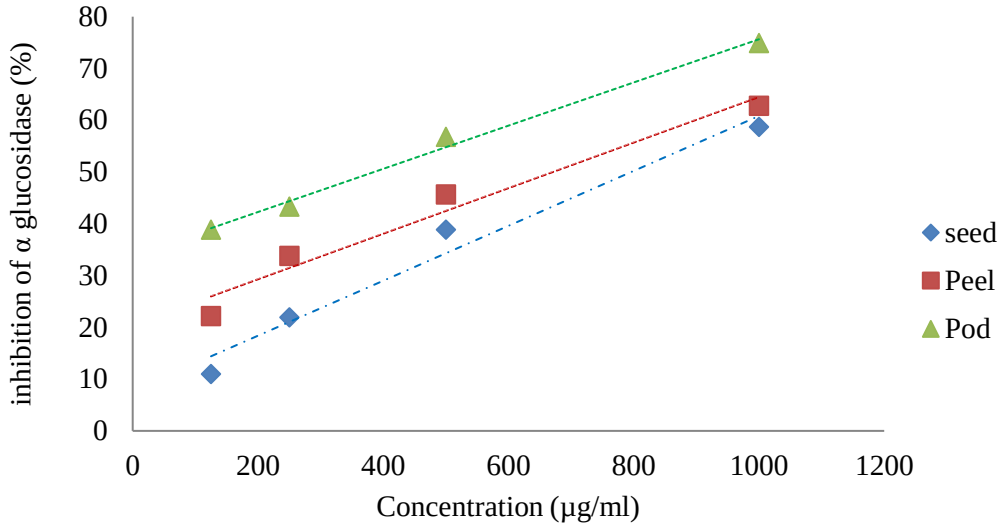


Figure 0: Inhibitory Effect of α-Glucosidase % (IC_{50} value).

The potential of α-glucosidase inhibitory of okra extracts was determined as shown in figure 3 and 4. There was significant difference between all aqueous extracts at the highest concentration (1000 mg/ml) and the lowest concentrations (125 mg/ml). Thus the aqueous extract of pod okra is significantly different from other concentrations of extracts, it had highest inhibitory potential on the enyzme.It was investigated that the extract with highest effectiveness of α-glucosidase and in which aqueous extract was also the most potent α-inhibitor with an IC_{50} value of 385.44 g/ml. The mode of α-glucosidase was determined by using graph, which displayed a mixed non-competitive inhibition of enyzme.

4 CONCLUSIONS

It can be concluded that okra extract can be used as a medicine to lower the blood glucose level by inhibiting the α-glucosidase and α-amylase enzyme activity. The inhibitory potential activity of okra extract againstα-glucosidase and α-amylase might be due to phyochemicals coponents present in the extract. In small intestine, the inhibitors of α-glucosidase delay the breaking down of glucose or carbohydrates and stop the postprandial blood glucose excursion in a person suffering from diabetes. To cure diabetes mellitus one method adopted which involves the inhibition of carbohydrate digeting enzyme such as α-glucosidase or α-amylase in the gastrointestinal glucose absorption therefore reducing the blood glucose level. This is an effort to find medicinal plant with increased effectiveness for alternative of drugs, medicinal plant have less cost and lesser side effects. This study suggests that the bioactive components in the extract compete with substrate to bind with the active site of enzyme which prevent the breaking down of carbohydrates (oligosaccharides to disaccharides).
The inhibitory activity of okra extract might be presence of phenolic component, flavonoids, and tannins in it. Previous studies on medicinal plant, which has α-amylase, and α-glucosidase inhibiting activity, these inhibitors belongs to the flavonoid class. From this study, it could be concluded that okra pod is rich source of protein, phenol and antioxidant. Pretreatment by soaking of okra enhancement of the yield of protein and antioxidant content. The antioxiadant activity was decreased as increase in temperature so could be advised that pretreatment for okra pod is at 40 °C for 30 min.

REFERENCES

- Adelakun, O. E., Oyelade, O.J, Ade-Omowye, Adeyemi, I.A & Van de Venter. (2009). Chemical composition and the antioxidative properties Nigerian Okra Seed *(Abelmoschus esculentus*).
- Arapitsas, P. (2008). Identification and quantification of polyphenolic compounds from okra seeds and skins. *Food Chem., 110: 1041-1045*

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72(1), 248-254.*
- Kazeem M. I., 1 Adamson J. O & Ogunwande I. A. (2013) "Modes of Inhibition of α-Amylase and α-Glucosidase byAqueous Extract of *Morinda lucida* Benth Leaf" BioMed Research *International Volume, Article ID 527570*
- Kukrić, Z. Z., Topalić-Trivunović, L. N., Kukavica, B. M., Matoš, S. B., Pavičić, S. S., Boroja, M. M., & Savić, A. V. (2012). Characterization of antioxidant and antimicrobial activities of nettle leaves *(Urticadioica L.). Actaperiodicatechnologica, (43), 257-272*
- Marinova, G., & Batchvarov, V. (2011). Evaluation of the methods for determination of the free radical scavenging activity by DPPH. *BulgarianJournalofAgriculturalScience, 17(1), 11-24.*
- Moyin- Jesu, E.I. (2007). Use of plant residues for improving soil fertility; pod nutrients, root growth and pod weight of Okra (Abelmoschus esculentus). Biocresour. *Technol., 98:2057-2064.*
- Oyelade, O.J., B.I.O. Ade-Omowaye & V.F. Adeomi, (2003). Influence of variety on protein, fat contents and some physical characteristics of okra seeds. *J. Food Eng., 57: 111-114.*

The Use of Predictive Models in the Context of Food Spoilage: The Case of White Pudding.

Emilie Cauchie[1]*, Nicolas Korsak[1], Mariem Ellouze[2], Laurent Delhalle[1], Bernard Taminiau[1], Fall Papa Abdoulaye[3] and Georges Daube[1]

[1] University of Liège, Fundamental and Applied Research for Animal & Health (FARAH), Faculty of Veterinary Medicine, Food Science Department, Quartier Vallée, Avenue de Cureghem 10, B-4000 Liège, Belgium.
[2] Nestlé Research Center, Food Safety Microbiology, route du Jorat 57, 1000 Lausanne, Switzerland.
[3] Quality Partner, rue Hayeneux 63, 4040 Herstal, Belgium.
*Corresponding authors (ecauchie@uliege.be and Mariem.Ellouze@rdls.nestle.com)

ABSTRACT

Food spoilage is a major issue for the food industry and consumers as products become unacceptable for human consumption leading to significant food waste and economic losses. This study combines the use of predictive microbiology and metagenetics in order to predict bacterial evolution in Belgian white pudding. The ecology of the product was studied at several times during the storage at constant temperatures, and under three different packaging (food wrap, modified atmosphere 30% CO_2 – 70% N_2 and vacuum packaging), by association of classical microbiological plate counting and 16S rRNA metagenetic analysis on each sample. The bacterial evolution could thus be deduced for the two major spoilage populations in the product, *Brochothrix thermosphacta* and *Pseudomonas* spp. The growth parameters were estimated using the nlsMicrobio package from R and then used to simulate the microbial behavior in dynamic conditions with three different softwares: ComBase, Sym'Previus and baranyi growth function in R. These results are compared with validation curves, obtained from these durability studies. A relatively good agreement was obtained between the validation data set and the simulations, showing that the approach combining the metagenetics and the simulations based on an accurate database is promising. Performance factors (bias and accuracy factors) indicated no significant structural deviation of the maximal growth rates simulations between observed and predicted values with R and Sym'Previus. An overestimation was mainly observed with R, while an underestimation was generally observed with Sym'Previus and ComBase. None of predictive simulations give an identical microbial curve that the validation data set, but all models show relatively good statistical fittings. This work gives a proof of concept on the feasibility to combine predictive models and metagenetics in order to predict bacterial evolution using different predictive tools. In the future, predictive models needed to be more accurate by taking into account as many growth parameters as possible.

KEYWORDS: Metagenetics, Predictive Microbiology, White Pudding, *Pseudomonas* spp., *Brochothrix thermosphacta*.

1 INTRODUCTION

Around a third of all food produced for human consumption on Earth is lost or wasted. In Europe, the losses of meat primary production represent 20%; and more than half of this occurs at the animal production, slaughtering, processing and distribution steps (Food and Agriculture Organization, 2011). Thus, food spoilage is a major issue for consumers and the food industry as products become unacceptable for human consumption, leading to significant food waste and economic losses. The reasons for food loss and waste are the contamination of the food matrix with spoilage bacteria. These features underline the importance of managing the microbiological quality of food products and raw materials. Fresh products provide a favorable environment for the survival and growth during storage of certain undesirable microorganisms (Garnier *et al.*, 2017).

Classical microbiology is not sufficient for a clear and complete understanding of the mechanisms that lead to the spoilage of food products. High throughput sequencing methods have allowed a more detailed and deeper description of bacterial species present in food and have revealed new bacterial species responsible for food spoilage. These techniques made it possible the identification and quantification of culturable and non-culturable organisms (Chaillou *et al.*, 2014, Parente *et al.*, 2016, Riquelme *et al.*, 2015). Among the culture-independent techniques, 16S rRNA metagenetics has emerged as a powerful tool for studying the bacterial composition of various ecosystems (Elizaquivel *et al.*, 2015, Parente *et al.*, 2016). However, few studies have described the evolution of a whole microbiota in a food matrix by taking into account the storage parameters (Ercolini *et al.*, 2011; Nieminen *et al.*, 2012).

Predictive microbiology is a research discipline of food microbiology that applies mathematical models to describe the growth and survival of microorganisms undergoing complex physical, chemical and biological changes during processing, transportation, distribution and storage of food (Huang, 2014; Pla *et al.*, 2015). Predictive microbiology can be used in combination with 16S rRNA metagenetics to improve food quality and reduce food losses. At present, various models are available to predict the effects of temperature, pH, aw, organic acids, modified atmosphere and other factors on microbial behavior (Li *et al.*, 2017; Martinez-Rios *et al.*, 2016). Unfortunately, few models describe the responses of spoilage bacteria owing to the fact that the majority of models have been developed for foodborne pathogens (Alfaro *et al.*, 2013; Membré and Lambert, 2008).

The objective of this work is to combine metagenetics results with predictive microbiological models on perishable foods under different environmental conditions through the case study of a typical Belgian pork meat product. We aim to (i) provide a proof of concept that combining plate count techniques and metagenetics analysis, with predictive modelling, can be a valuable tool to better explore the evolution of microorganisms present in perishable food within different environmental conditions, (ii) and to use these results to predict the dynamics of the evolution of the dominant microbial community in food products, as spoilage bacteria, and compare these results with some of the available models and software applications such as ComBase, Sym'Previus and growth packages from the open source R software.

2 MATERIAL AND METHODS

2.1 White Pudding Sample Preparation

Fresh white pudding (WP) packed under air with a food wrap film was obtained from a local Belgian manufacturer. The water activity of this product was 0.98 ± 0.02 and the pH value was 6.00 ± 0.20. WP samples were put (300 g) in three different packaging, in triplicate: in a tray under modified atmosphere (MAP, CO2 30% / N2 70%) using packaging wrap; in a tray under food wrap packing (FW) (cling film); and in vacuum packaging (VP) using bags.

2.2 Durability Studies

Durability studies were performed according to the standard for implementing microbiological durability tests of chilled perishable and highly perishable foodstuffs (AFNOR, NF V01-003, 2010). WP samples in FW packaging were stored during 12 days of shelf life and during 28 days under MAP and VP packaging, at constant temperature of 4°C, 8 °C or 12 °C in temperature-controlled incubators. At each day of the trials, total psychrotrophic viable counts and 16S rRNA metagenetics analysis were carried out on all the samples.

2.2.1 Plate Count Enumeration

Each day during the storage period, 25 g of product were put into a Stomacher bag with a mesh screen liner under aseptic conditions. Physiological water (225 mL) was automatically added to each bag and the samples were homogenized for 2 min in a Stomacher. From this primary suspension, decimal dilutions in peptone water (1.0 g/L peptone, 8.5 g/L sodium chloride) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for

each analysis in triplicate. Total viable counts (TVC) for the psychrotrophic flora were performed on plate count agar (PCA) after incubation for 48 h at 22 °C.

2.2.2 Total DNA Extraction

Bacterial DNA was extracted from each primary suspension, previously stored at – 80°C, using the DNeasy Blood & Tissue DNA Extraction kit. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer. The quality and quantity of the products were confirmed by Picogreen double-stranded DNA (dsDNA) quantitation assay. DNA samples were stored at – 20°C until used for 16S rRNA amplicon pyrosequencing.

2.2.3 Bacterial 16S rRNA Gene Amplification and Barcoded Pyrosequencing

16S rRNA PCR libraries targeting the V1-V3 hypervariable region were generated. Primers E9-29 and E514-430, specific for bacteria, were selected for their ability to generate the lowest amplification bias relative to amplification capability among the various bacterial phyla. The oligonucleotide design included 454 Life Sciences A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs), fused to the 5' end of each primer. The amplification mix contained 5 units (U) of FastStart high fidelity polymerase (Roche Dianostics, Vilvoorde, Belgium), 1 x enzyme reaction buffer, 200 µM deoxynucleotide triphosphates (dNTPs) (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng of genomic DNA in a final volume of 100 µL. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 sec at 94 °C), annealing (45 s at 56 °C) and extension (60 s at 72 °C), with a final elongation step (8 min at 72 °C). These amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). The quality and quantity of the products were assessed by Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). Equal amounts of each of the PCR products were pooled and subsequently amplified by emulsion PCR. Pyrosequencing was performed with the Illumina sequencer (Illumina, Eindhoven, Netherlands) (2 x 300 bp). A mean of 60,000 reads for all days and all temperatures were analyzed for each FW packaging condition.

2.3 Bioinformatics Analysis

The 16S rRNA gene sequence reads were processed with MOTHUR. The sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer. The obtained read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences implemented in MOTHUR. The final reads were clustered into operational taxonomic units (OTUs), using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA data set 111, using a BLASTN algorithm (Delcenserie *et al.*, 2014). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (< 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Delcenserie *et al.*, 2014). As used by Cauchie *et al.* (2017) (Eq.1), the percentage of each OTU was finally converted as a proportion of the total viable count, obtained by classical microbiological analysis, in order to obtain estimate counts for each species (in log CFU/g).

$$C_{\text{bacterial species}} = (C_{\text{total flora}} \times P_{\text{reads of bacterial species}}) / 100 \quad (1)$$

Where $C_{\text{bacterial species}}$ is the estimated abundance concentration in the sample (log CFU/g), $C_{\text{total flora}}$ is the bacterial concentration per samples in the PCA analysis (log CFU/g), and $P_{\text{reads of bacterial species}}$ is the proportion of reads for the bacterial species per samples in the metagenetic analysis (expressed in % of the total number reads in the sample).

According to this, a lower diversity of results was observed in the FW packaging and we selected this packaging to carry out the proof of concept and run the predictive microbiology analysis only on those results.

2.4 Predictive Microbiology: Fittings

Growth kinetics of the dominant spoilage flora were deduced from TVC counts and the OTU proportions. The primary growth model of Baranyi and Roberts (1994) was used to fit the data and estimate the growth parameters: lag-time (lag), initial bacterial concentration (N_0), maximal bacterial concentration (N_{max}) and maximal growth rate (μ_{max}). A reparametrized version of the square root secondary model (Eq. 2) was then used to assess the effects of temperature on the growth rates.

$$\mu_{max} = \mu_{\text{ref}} \left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \quad (2)$$

Where μ_{ref} is the reference growth rate obtained at T_{ref} = 20°C, T is the temperature and T_{min} is the minimal temperature for growth found in the scientific literature. All fittings were performed using the nlsMicrobio package from the open source R software (R Core Team, 2016). These fittings were realized only on duplicate data sets from the durability study, the third dataset was kept for validation.

2.5 Simulation and validation

Simulations were run at 4 °C, 8 °C and 12 °C for each species, using three independent software programs: the ComBase Predictor, Sym'Previus and R software. To run the simulations, information about the initial micro-organism load (N_0), the temperature and the physico-chemical characteristics (pH, a_w) were set to the observed values during the durability studies and introduced in the different software. Table 1 gives an overview of the simulations hypothesis only for *Brochotrix thermosphacta* as a proof of concept in this article.

Table 1: Simulation hypothesis used for *Brochothrix thermosphacta* with the three predictive software programs

	Temperature (°C)	pH	a_w	N_0 (log CFU/g)	N_{max} (log CFU/g)	Lag (h)	μ_{max} (1/h)	Phys. state
ComBase	4, 8, 12	6.00	0.98	0.54	- [a]	- [a]	- [b]	4.4×10^{-4}
Sym'Previus	4, 8, 12	6.00	0.98	0.54	7.16, 7.57, 8.39	30 [c]	0.09 [c]	- [a]
R	4, 8, 12	- [a]	- [a]	0.54	7.16, 7.57, 8.39	48,30,25	0.07,0.14, 0.25 [d]	- [a]

[a] not taken into account in the model, [b] calculated by polynomial model of the tool, [c] calculated by model of the tool for a reference at 8°C, [d] model based on the reparametrized version of the square root secondary model.

The analysis of covariance (ANCOVA) test was used to evaluate if the results were significantly different between the validation data set and each of the simulation results at each time using R software. All tests were considered as significant for a p-value of < 0.05.

The predicted growth rate parameters were evaluated using the bias factor (Bf) and accuracy factor (Af) (Lee *et al.*, 2014), using Eqs. 3 and 4 respectively, in R software:

$$B_f = 10^{\frac{\sum_{i=1}^{n} \log\left(x_{predicted} / x_{observed}\right)}{n}} \quad (3)$$

$$A_f = 10^{\frac{\sum_{i=1}^{n}\left|\log\left(x_{predicted}/x_{observed}\right)\right|}{n}} \quad (4)$$

Where $x_{predicted}$ is the value obtained from simulations with R, ComBase and Sym'Previus, respectively, and $x_{observed}$ is the value from the validation data set.

The bias factor (Bf) is a measure of average variation between the observed and predicted values. The Af indicates the spread of results about the prediction by disregarding whether the difference is positive or negative. A value factor close to 1 indicated that the model used in this work has high capability to fit the number of bacteria on WP.

3 RESULTS

3.1 WP Ecosystem Characterization and Identification of Major Species

Figure 1 shows the relative abundance results obtained by metagenetics analysis for FW conditions at 4 °C (a.), 8 °C (b.) and 12 °C (c.).

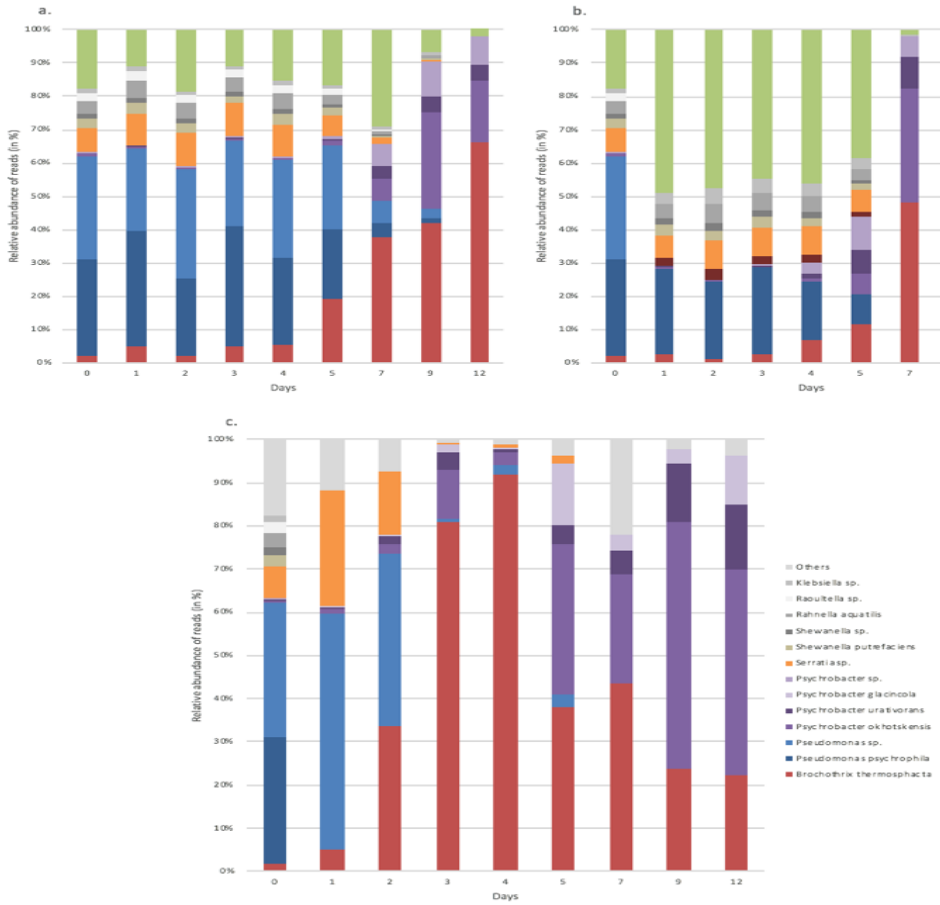


Figure 1: Bar chart detailing the relative abundances of the major bacterial species naturally present in white pudding samples in food wrap packaging at 4°C (a.), 8 °C (b.) and 12 °C (c.). Only the most abundant bacterial species are in colour. Others species represent the total percentage of reads less than 1%.

The distribution of read percentages shows that the major bacterial spoilage microorganisms were *Brochothrix thermosphacta*, *Pseudomonas* spp., *Psychrobacter* spp., *Serratia* spp. and *Shewanella* spp. For all temperature conditions *B. thermosphacta* and *Psychrobacter* spp. were the major bacterial species at the end of the product shelf life and therefore used in the rest of the study.

3.2 WP kinetics and Fittings Results

Figure 2 shows the PCA results from naturally contaminated Belgian white pudding at different temperatures (4, 8 and 12 °C) in FW packaging, stabilized between 8.0 and 9.0 log CFU/g.

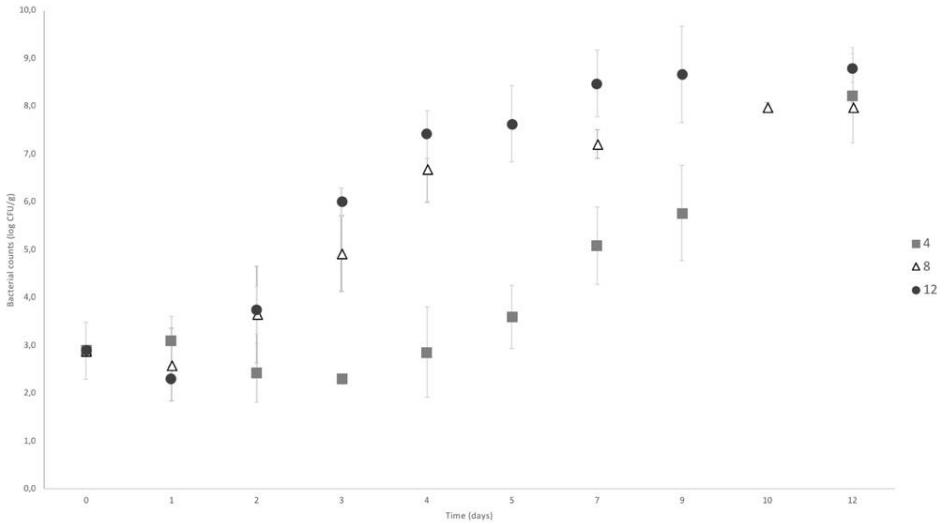


Figure 2: Enumeration of the total psychrotrophic aerobic microorganisms from naturally contaminated Belgian white pudding in food wrap packaging and stored at 4°C (squares), 8 °C (diamonds) and at 12 °C (triangles).

The bacterial growth parameters also showed different dynamic changes depending on storage temperature: a high storage temperature is correlated to a high growth rate during exponential phase and a lower lag-time.

3.3 FW Packaging Simulations and Validation

Figure 3 shows the comparison between the observations from the validation data set obtained in the durability studies and simulations by the three software programs for *Brochothrix thermosphacta* as an example.

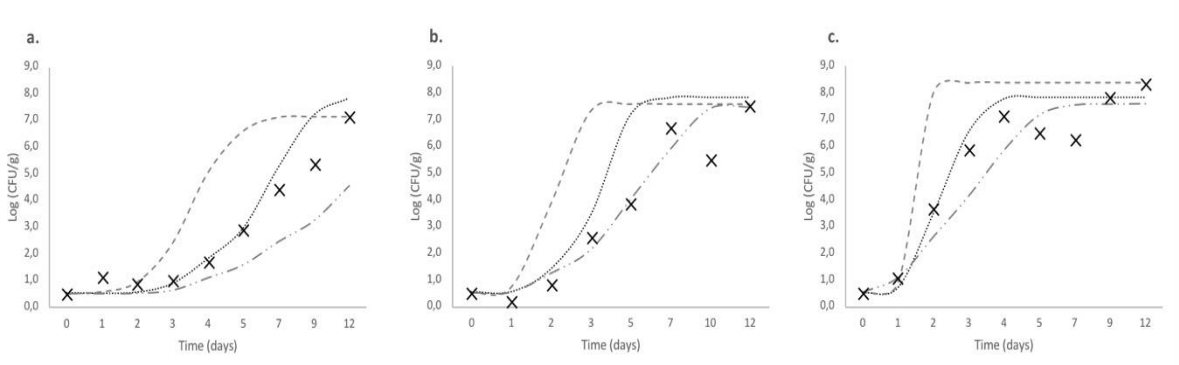


Figure 3: Observed (x) and predicted results (lines) in FW for *Brochothrix thermosphacta*, at 4 (a.), 8 (b.) and 12°C (c.). The predicted data obtained by the three software tools are indicted by lines: ComBase (•••••••, dotted line), Sym'Previus (— · · —, dashed line) and the open source R software (– – –, broken line).

For the overall plot, a relatively good agreement is obtained between the observations and the simulations, showing that the approach combining the metagenetic and simulations based on an accurate database is promising. However, higher variability could be observed between simulations.

The correlation of the observed values obtained by the validation dataset with the predicted values obtained by simulations were performed for growth rates (Table 2).

Table 2: Correlation of the predicted growth rate with the observed values for *Brochothrix thermosphacta* in FW using Sym'Previus, ComBase and the open source R software.

Models	Growth parameters			Evaluations	
	Temp. [a]	Obs. μ_{max} [b]	Pred. μ_{max} [c]	B_f [d]	A_f [e]
ComBase	4	0.07	0.05	0.845 *	1.183
	8	0.10	0.09	0.949	1.054
	12	0.22	0.13	0.769 *	1.301
Sym'Previus	4	0.07	0.04	0.756 *	1.323
	8	0.10	0.09	0.949	1.054
	12	0.22	0.15	0.826 *	1.211
R software	4	0.07	0.07	1.000	1.000
	8	0.10	0.14	1.183	1.183
	12	0.22	0.25	1.066	1.066

[a] temperature (°C), [b] the observed maximal growth rate (μ_{max}, 1/h), [c] the predicted maximal growth rate value (μ_{max}, 1/h), [d] bias factor, [e] accuracy factor, * unacceptable model performances < 0.85 or >1.25 (Mejholm and Dalgaard, 2015).

As previously suggested for spoilage bacteria, the performance of a new model can be considered acceptable for B_f values in the range of 0.85 to 1.25 for lactic acid bacteria (Mejholm and Dalgaard, 2015). The most unacceptable performances were observed with ComBase and Sym'Previus simulations. Perfect fit of 1 was mainly observed with R software.

4 DISCUSSION

In this experiment, the total microbial load was estimated by using the plate count agar medium to observe the development of a whole ecosystem on Belgian white pudding. Although this method is widely used to follow the microbial evolution in food matrix, these results are not informative enough to describe bacterial communities. Metagenetics tools could be used as a supplementary technique to elucidate the detailed dynamics of the microbial population during spoilage, as shown in our results. However, this technique is not sufficient because it gives only relative results, which need to be associated with quantitative estimates, to assess the microbial load. In this study, the growth of bacteria was assessed daily at the same time, by combining classical microbiology and 16S rRNA metagenetics analysis, to provide a clear picture of the microbial community, to closely follow the obtaining of quantitative results for each bacterium during aging tests, and to determine the dominant bacterium within different environmental conditions. By integrating metagenetics with traditional microbiological analysis it was possible to extend this view of a highly quantitative characterization of dynamic changes that occur during chilled storage. These data may also contribute to set a database on food ecosystem composition.

In addition to predictive microbiology, these data enabled us to simulate the growth of each dominant bacterium. In this work, the data used as input for predictive models only concerned *B. thermosphacta* and *Pseudomonas* spp. as spoilage bacteria, with three different available models and predictive software. *B. thermosphacta* was then used as a proof of concept. The comparison of simulations showed a good fitting with validation curves obtained from durability studies. But it should be noted that an overestimation was mainly observed with the baranyi growth function in R, while an underestimation was more frequently observed with Sym'Previus and ComBase. These observations may be influenced by the condition of storage and/or the bacterial species. None of the predictive simulations gave an identical microbial curve to the validation kinetics obtained by the aging-test, even if all of the models showed good statistical fits with these data. The best performance factors were mainly observed with R and Sym'Previus. According to these results, some hypothesis can explain these differences. Indeed, the simulations did not take into account the effect of the competitive flora present in the product and only focused on one or two targeted species. Indeed, our study only concerned the two major bacterial species, *Brochothrix thermosphacta* and *Pseudomonas* spp., but as mentioned in the results section, others species are also present such as *Psychrobacter* spp. So, significant statistical differences for simulations are probably due to an interaction between bacterial species, compared to the evolution of each isolated bacterium in the same food matrix. On this basis, it would be worth

including the possibility of considering microbial interactions between different types of microorganisms, to better predict microbial growth or inactivation in white pudding, and in complex food ecosystems (Tenenhaus-Aziza and Ellouze, 2015). Furthermore, as shown in the simulation hypothesis, the growth parameters used in the three software are not the same, which could explain the variability between simulations. Indeed, the food matrix may have an effect on the bacterial evolution, but only ComBase and Sym'Previus software applications took into account the intrinsic parameters of the Belgian white pudding, such as pH and water activity, which can explain why the R simulations sometimes showed an overestimation of the kinetics. It is possible to add intrinsic parameters with R simulations, but more cardinal values about the spoilage microorganisms are needed (T_{min}, T_{max}, T_{opt}, $a_{w(min)}$, $a_{w(opt)}$, $a_{w(max)}$, pH_{min}, pH_{opt}, pH_{max}, …). Finally, all bacteria chosen were in an internal database of ComBase but it was not possible to select a precise bacterial strain or food matrix for these simulations.

5 CONCLUSIONS

Compared to culture-based methods on selective media and previous independent culture techniques, metagenetics analysis combined with predictive microbiology gave more valuable information, and its use could be considered as a technique for quality control or for accurately determining shelf life. Microbiological ecology studies have shown that the microbiota of food is much more diverse than the cultivated group of bacteria studied by the use of culture media. The use of these new technologies will open a new era for modelling and predictive microbiology.

6 ACKNOWLEDGEMENTS

Our sincere thanks go to Proof-Reading Service (Devonshire Business Center, United Kingdom) for the support in English editing of the manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

- AFNOR (2010). Hygiène des aliments, Lignes directrices pour la réalisation de tests de vieillissement microbiologique, Aliments périssables et très périssables réfrigérés, NF V01-003. France : Association française de normalisation.
- Alfaro, B., Hernandez, I., Le Marc, Y., & Pin, C. (2013). Modelling the effect of the temperature and carbon dioxide on the growth of spoilage bacteria in packed fish products. *Food Microbiology*, *29*, 429-437.
- Baranyi, J., & Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, *23*, 277-294.
- Cauchie, E., Gand, M., Kergourlay, G., Taminiau, B., Delhalle, L., Korsak, N., & Daube, G. (2017). The use of the 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding. *International Journal of Food Microbiology*, *247*, 70-78.
- Chaillou, S., Chaulot-Talmon, A., Caekebeke, H., Cardinal, M., Christieans, S., Denis, C., Desmonts, M.H., Dousset, X., Feurer, C., Hamon, E., Joffraud, J.J., La Carbona, S., Leroi, F., Leroy, S., Lorre, S., Macé, S., Pilet, M.F., Prévost, H., Rivollier, M., Roux, D., Talon, R., Zagorec, M., &Champomier-Vergès, M.C. (2014). Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. *ISME Journal*, *9*, 1105-1118.
- Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., Roussey, D., Korsak, N., & Daube, G. (2014). Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. *Journal of Dairy Science*, *97*, 6046-6056.
- Elizaquivel, P., Pérez-Cataluna, A., Yépez, A., Aristimuno, C., Jiménez, E., Cocconcelli, P. S., Vignolo, G., & Aznar, R. (2015). Pyrosequencing vs. culture-dependent approaches to analyze

lactic acid bacteria associated to chicha, a traditional maize-based fermented beverage from Northwestern Argentina. *International Journal of Food Microbiology, 198,* 9-18.

- Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A., Laghi, L., Mauriello, G., Guerzoni, M.E., & Villani, F. (2011). Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. *Applied and Environmental Microbiology, 77,* 7372-7381.
- Food And Agriculture Organization of the United Nations (FAO), 2011. Global food losses and food waste – extent, causes and prevention. Romanticism.
- Garnier, L., Valence, F., Pawtowski, A., Auhustsinava-galerne, L., Frotté, N., Baroncelli, R., Deniel, F., Coton, E., & Mounier, J., 2017. Diversity of spoilage fungi associated with various French dairy products. *International Journal of Food Microbiology, 241,* 191-197.
- Huang, L. (2014). IPMP 2013 – A comprehensive data analysis tool for predictive microbiology. *International Journal of Food Microbiology, 171,* 100-107.
- Lee, Y.J., Jung, B.S., Yoon, H.J., Kim, K-T., Paik, H-D., & Lee, J-Y. (2014). Predictive model for the growth kinetics of Listeria monocytogenes in raw pork meat as a function of temperature. *Food Control, 44,* 16-21.
- Li, L., Cepeda, J., Subbiah, J., Froning, G., Juneja, V.K., & Thippareddi, H. (2017). Dynamic predictive model for growth of Salmonella spp. in scrambled egg mix. *Food Microbiology, 64,* 39-46.
- Martinez-Rios, V., Ostergaard, N.B., Gkogha, E., Rosshaug, P.S., & Dalgaard, P. (2016). Modelling and predicting growth of psychrotolerant pseudomonads in milk and cottage cheese. *International Journal of Food Microbiology, 216,* 110-120.
- Mejholm, O., & Dalgaard, P. (2015). Modelling and predicting the simultaneous growth of Listeria monocytogenes and psychrotoleran lactic acid bacteria in processed seafood and mayonnaise-based seafood salads. *Food Microbiology, 46,* 1-14.
- Membré, J.M., & Lambert, R.J.W. (2008). Application of predictive modelling techniques in industry: from food design up to risk assessment. *International Journal of Food Microbiology, 128,* 10-15.
- Nieminen, T.T., Koskinen, K., Laine, P., Hultman, J., Säde, E., Paulin, L., Paloranta, A., Johansson, P., Björkroth, J., & Auvinen, P. (2012). Comparison of microbial communities in marinated and unmarinated broiler meat by metagenomics. *International Journal of Food Microbiology, 157,* 142-149.
- Parente, E., Cocolin, L., De Filippis, F., Zotta, T., Ferrocino, I., O'Sullivan, O., Neviani, E., De Angelis, M., Cotter, P.D., & Ercolini, D. (2016). FoodMicrobionet: a database for the visualisation and exploration of food bacterial communities based on network analysis. *International Journal of Food Microbiology, 219,* 28-37.
- Pla, M.L., Oltra, S., Esteban, M.D., Andreu, S., & Palop, A. (2015). Comparison of primary models to predict microbial growth by the plate count and absorbance methods. *Biomed Research International, 2015,* 1-14.
- R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/
- Riquelme, C., Câmara, S., Enes Dapkevicius, M. de L.N., Vinuesa, P., da Silva, C.C.G., Malcata, F.X., & Rego, O.A. (2015). Characterization of the bacterial biodiversity in Pico cheese (an artisanal Azorean food). *International Journal of Food Microbiology, 192,* 86-94.
- Tenenhaus-Aziza, F., & Ellouze, M. (2015). Software for predictive microbiology and risk assessment: a description and comparison of tools presented at the ICPMF8 Software Fair. *Food Microbiology, 45,* 290-299.

Effects of Drying Temperature on Color and Total Curcuminoids Contents in Turmeric

Nilobon Komonsing[1], Pramote Khuwijitjaru[1], Marcus Nagle[2], Busarakorn Mahayothee[1]*, Joachim Mueller[3]

[1]Department of Food Technology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakhon Pathom, Thailand
[2]Agricultural Research and Development Program, Central State University, Ohio, USA
[3]Institute of Agricultural Engineering, Universität Hohenheim, Stuttgart, Germany
*Corresponding Author (busarakornm@yahoo.com)

ABSTRACT

Turmeric (*Curcuma longa* L.) is a culinary and medicinal herb widely grown in Asian countries. Its rhizome is a good source of curcuminoids which play an important role as orange-yellow color pigment, antioxidant, and anti-inflammatory agent. Turmeric is generally processed into dried slices and powder for extending shelf-life. In this study, the influence of drying temperature on color and total curcuminoid content was investigated. Fresh turmeric rhizomes at the maturity of 9 months were sliced to a thickness of 3.5 mm, and then cut into a rectangular shape of 3.5 × 40 × 8 mm before drying. Drying experiments were conducted using a laboratory-made hot air dryer in an over- and under-flow mode. Drying was carried out at five temperatures (40, 50, 60, 70, and 80 °C). Air velocity and specific humidity of drying air were fixed at 0.5 m/s and 25 g water/ kg dry air, respectively. The fresh samples were dried until the moisture content was lower than 12%. Fresh and dried samples were taken for color measurement (L*, a*, b*, C, and h°) and total curcuminoid content analysis. For color determination of dried samples, dried samples were ground into powder prior to analysis by a colorimeter. The drying times used for drying at 40, 50, 60, 70, and 80 °C were 37, 26, 9.7, 6.8, and 3.6 h, respectively. It was found that with a very large difference in drying times, drying temperature did not obviously influence the color values of dried products. However, b*, C and h° values of turmeric powder obtained from 70 °C were significantly different from those of 40 and 50 °C ($p < 0.05$). In addition, drying temperature did not show significant effects on total curcuminoid contents of the dried samples ($p > 0.05$). Thus, turmeric slices using a tray dryer could be done at higher temperature for accelerating the drying process without an adverse impact on the color and bioactive compounds of dried products.

KEYWORDS: Herbs, Spices, Dehydration, Yellow Pigments, Curcumin

1 INTRODUCTION

Turmeric (*Curcuma longa* L.) is one of the most prominent herbs and spices that contains natural bioactive compounds. It belongs to the family of Zingiberaceae, which is a plant native to Southern Asia (López-Tobar *et al.*, 2012) before spreading throughout tropic and subtropical regions of the world. The cultivation of turmeric found mainly in India, Pakistan, China, Haiti, Jamaica, Peru, Taiwan, Indonesia, Madagascar, and Thailand (CBI, 2016). According to Agricultural Extension Department (2016), the production of turmeric in Thailand was about 3,550 tons. Turmeric rhizome is can be used as spices, food additives, colorants, cosmetics, food supplement and medicine in various forms including fresh, powder, extract, and essential oil. Major active compounds in turmeric rhizome are curcuminoids which comprise of curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) and its analogs, demethoxycurcumin, and bisdemethoxycurcumin which give its characteristic orange-yellow color (E100(ii)). Over the past decades, numerous studies have reported the important roles of turmeric and curcuminoids in health promoting including antioxidant (Gómez-Estaca *et al*., 2017), anti-inflammatory (Zhang *et al.*, 2015), anticancer (Patil et al., 2018), antimicrobial (de Oliveira *et al.*, 2018), antitumoral (Tirca *et al*., 2017) and antiviral activities (Mathew & Hsu, 2018).

Tumeric rhizome is usually dried to extend its shelf-life. Sun drying is economical on a large scale since it is cheap and simple method. However, this method has many disadvantages such as

deterioration from pests and contamination of dust. Moreover, light may lead to poor qualities in term of color loss, and curcuminoids degradation (Mondal *et al*., 2016). Solar dryer and hot air dryer have been employed to replace sun drying for producing dried turmeric. Karthikeyan & Murugavelh (2018) dried turmeric rhizomes in a solar tunnel dryer and found that the drying time was 12 h while it took 43 h under open sun drying. Lakshmi *et al.*, (2018) studied the effects of solar drying on color, antioxidant activity, total phenolic contents, and total flavonoid contents of black turmeric slices. It was found that the samples obtained from solar dryer showed higher antioxidant activity (28.33 μmol of trolox equivalent/g sample) compared to those from sun drying (22.04 μmol of trolox equivalent/g sample). Enhancing of curcuminoids degradation can occur via thermal treatment (Siddiqui, 2015). However, study on the effect of drying temperature on qualities of turmeric slices is still limited. Thus, the objective of this study was to investigate the influence of drying temperature on color and total curcuminoid content in turmeric slices.

2 MATERIALS AND METHODS

2.1 Sample Preparation

Two lots of turmeric rhizomes with the maturity of 9-10 months after planting from Surat Thani province, Thailand were used. This maturity is commonly used for traditional medicine production. Uniform size of rhizomes were cleaned and packed in net bags. They were carried to the laboratory in Stuttgart, Germany and then kept at 11.0 ± 0.4 °C and the relative humidity of 90.4 ± 11.6% before drying experiments.

Fresh turmeric rhizomes were washed under running tap water prior to drying. They were air dried and measured for weight, length, and diameter of the rhizomes (n = 40) from each experiment lot using a digital balance (BS 4202S, Sartorius, Göttingen, Germany) and digital vernier caliper, respectively. After that, they were sliced into a thickness of 3.5 mm. The slice samples were drilled using a special made cutter into a size of 3.5 × 40 × 8 mm. Approximately 200 pieces of turmeric sample (*ca*. 250 g) were placed on an aluminum wire tray in a single layer.

2.2 Drying Experiment

Drying experiments were conducted using a laboratory-made hot air dryer at the Institute of Agricultural Engineering, the University of Hohenheim, Stuttgart, Germany (Fig. 1). The dryer consisted of four main units, 1) an air flow control unit, 2) an air conditioning unit, 3) a heating control unit, and 4) over- and under- flow drying compartment which are controlled by PID controller. First, fresh air from environment enters the drier through a thermostat-controlled water bath containing sprayed Rasching-ring bed. After that, air with high humidity was preheated to approximately 1°C below the target temperature, and finally it was secondary heat to the target temperature (Argyropoulos *et al.*, 2011). The drying experiments were performed at five temperatures including 40, 50, 60, 70 and 80 °C with an air velocity of 0.5 m/s and the specific humidity of drying air at 25 g H_2O/kg air using an over- and under-flow mode. The dryer was started about 2 h before each drying experiment to achieve steady state conditions. During drying experiment, temperature, air velocity, and relative humidity of drying air were recorded automatically by a connected industrial computer using PLC software. A main sample tray was placed at the center of drying chamber where a load cell (Flintec, type PC6, Vasteras, Sweden) was installed. For drying at 40 and 50 °C, they were weighed automatically every 15 min, while every 10 min for drying at 60, 70, and 80 °C. Samples were dried until the mass of the products reached a constant value at the moisture content about 12%. After that, dried samples were randomly taken for color measurement, then kept in a jar for 12 h, vacuum packed in aluminum foil bag and stored at -22 °C prior to total curcuminoid content analysis. The drying experiments were conducted in duplicate.

Figure 1: A laboratory-made hot air dryer (left) and over- and under- flow drying compartment (right).

2.3 Color Measurement

Color values of fresh and dried samples were measured using a Chroma Meter (CR-100, Minolta Camera, Japan). White calibration plate D65 (Y = 87.5, x = 0.3180 and y = 0.3355) were used for standardization. The color values in CIE L* a* b* were obtained. For the fresh samples, 30 pieces of turmeric slices were randomly measured at 2 positions on each piece. For dried tumeric, sample were ground into powder using a blender, put in a sample holder before measurement. Chroma (*C*), hue angle ($h°$) and total color change (ΔE) were calculated using the following equations:

$$C = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$h° = tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (2)$$

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (3)$$

Where, Δ refers to difference of each parameter between fresh and dried samples.

2.4 Moisture Content and Water Activity

Moisture content (*MC*) of fresh samples and the samples after drying was measured using vacuum drying method as described by de Melo & Almeida-Muraduan (2011) with some modifications. Fresh small pieces (*ca.* 1 mm) and dried powder (3–5 g) were dried at 50 °C, 2 kPa for 24 h. For calculation of total curcuminoid content in dry basis, moisture content of sample was determined using conventional hot-air oven drying method at 105 °C (AOAC, 1999).

Water activity (a_w) was measured using a water activity meter (AW-DIO, Rotronic, Switzerland) after equilibrating 2 g of sample for 20 min in a thermostatic cell at 23 °C.

2.5 Determination of Total Curcuminoid Content

Fresh sample (3 g) or dried powder (2 g) was mixed with 20 mL of methanol in 50 mL centrifuge tube using a vortex mixer (G560E, Scientific Industries, NY, USA) for 15 s. After that, they were extracted using an ultrasonic bath (Sonorex Digital 10 P, Bandelin, Berlin, Germany) for 30 min (temperature of water was lower than 35 °C). The mixture was filtered through Whatman No.4 filter paper. The residue was re-extracted two times with 15 mL of methanol in the ultrasonic bath for 30 min. Crude extract was pooled and adjusted to 50 mL in volumetric flask. The extract was kept in an amber vial at -22 °C for one night prior to curcuminoid contents determination. All the extractions were performed in triplicate. The crude extract was determined for total curcuminoid contents by UV-Vis

spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Waltham, MA, USA) according to Martins *et al.,* (2013) with some modifications. The wavelength was set at 425 nm. A stock solution of standard curcumin (purity ≥ 98%, Merck, NY, USA) was prepared in methanol at a concentration of 250 mg/L. Stock solution was diluted to 6 concentrations (0, 1.0, 2.5, 5.0, 6.0, 8.0, 10.0 and 12.0 mg/L) for preparing a calibration curve. Total curcuminoid content was expressed as mg curcumin/g dry matter.

2.6 Statistical Analysis

Analysis of variance and Duncan's Multiple Range Test were performed using SPSS version 17 (SPSS, Chicago, IL, USA) to evaluate the difference of color parameters and total curcuminoids contents using sample lots as blocks. The results were assessed at a probability level of ≤ 0.05.

3 RESULTS AND DISCUSSION

3.1 Quality of Fresh Turmeric

Physical properties of fresh rhizomes before drying are showed in Table 1. Data indicate homogeneity with low dispersion of the samples. The average weight, length, and diameter of turmeric rhizomes of this study were similar to reported from De Lima *et al.,* (2016) with the weight, length and diameter were 16.41, 67.79, and 15.80 mm, respectively. Homhual (2010) reported that the length and diameter of fresh turmeric rhizomes should be about 40-70 and 10-18 mm.

Table 1: Properties of fresh turmeric rhizomes (n = 40)

Parameters	Average ± SD
Rhizome weight (g)	15.17 ± 1.05
Rhizome length (mm)	84.45 ± 1.56
Rhizome diameter (mm)	13.91 ± 0.63
Color parameters	
*L** (lightness)	58.61 ± 0.14
*a** (+redness)	34.86 ± 0.88
*b** (+yellowness)	61.92 ± 0.79
C	71.29 ± 1.06
h°	60.51 ± 0.51
Moisture content (% wet basis)	77.68 ± 2.26
Water activity (23 °C)	0.693 ± 0.004
Total curcuminoid content (mg curcumin/g fresh weight)	17.61 ± 1.05

The samples showed positive values of a* and b* and h° about 60, indicating characteristic orange-yellow of fresh turmeric. The average moisture content of fresh rhizomes was 77.68% which was lower than 91.50% shown in the study by Hirun *et al.,* (2014). They suggested that the disparity of moisture content could be due to harvest season. From Table 2, an average total curcuminoid content of fresh rhizome was 1.76% (wet basis). The result agreed with Sikkhamondhol *et al.,* (2009) who reported the total curcuminoid content in fresh turmeric in Thailand of 1.05% (wet basis).

3.2 Effect of Drying Temperatures on Color

In this study, turmeric slices were dried until the moisture content was approximately 12%. The drying time at 40, 50, 60, 70, and 80 (°C) were 3 h 33 min, 6 h 17 min, 9 h 43 min, 26 h and 37 h, respectively. Table 2 shows moisture content and water activity of dried turmeric from each drying temperature. Drying at 40 and 50 °C could not remove the moisture content to less than 12% due to the equilibrium moisture content between hot air and the sample surfaces. The relative humidity of drying air at 40 and 50 °C were 53.2 ± 2.5 and 33.3 ± 2.1%.

Table 2: Moisture content (MC) and water activity (a_w) of dried products obtained from various drying temperatures.

Temperature (°C)	MC (%)	a_w
40	13.26 ± 0.55^{a}	0.444 ± 0.039^{a}
50	12.98 ± 0.82^{b}	0.418 ± 0.112^{b}
60	11.07 ± 0.81^{c}	0.248 ± 0.001^{d}
70	10.58 ± 0.62^{c}	0.262 ± 0.020^{c}
80	11.19 ± 0.66^{c}	0.263 ± 0.006^{c}

Data were expressed as mean ± SD (n = 2).
* Significant differences ($p < 0.05$) within a column denoted by different superscript letters.

Because turmeric is often processed into powder. Color of powder is more important than the color of dried piece. Dried turmeric were ground and measured for *L**, *a**, *b**, *C* and *h*° (Fig. 2). *a**, *b** and *C* values decreased after drying while L* values did not significantly changed. The color of fresh turmeric slices changed from bright orange-yellow to orange-brown after drying. Monisha *et al.,* (2016) reported that *a** and *b** of turmeric decreased after microwave drying at all power levels (180, 360, 540, 720 and 900 W) and demonstrated that exposure time (24, 48, 72, 96 and 120 s) did not significantly affect the total color difference of dried turmeric. The optimum condition of minimal change in *L**, *a**, *b** and ΔE values was drying at 368.8 W for 45.68 s. In this study, it was found that the drying temperature did not obviously influence the color values of dried products. *L**values of dried products were in the range of 50.37 – 54.48. The results were similar to Prathapan *et al.,* (2009) who showed *L**values of boiled turmeric rhizomes at 60 – 80 °C was in the range of 52 - 60. Hirun *et al.,* (2014) reported that increasing of drying time from 10 to 30 min and heating power of microwave from 2,400 – 4,000 W significantly increased the *L** value of dried turmeric due to inactivation of polyphenol oxidase and peroxidase. *b**, *C* and *h*° values of turmeric powder obtained from 70 °C were significantly different from those of 40 and 50 °C ($p < 0.05$). These three color values of powders from 70 °C were the least affected which indicated better color of dried product. However, total color difference (ΔE) values were not significantly different in each drying temperature.

3.3 Effect of Drying Temperatures on Total Curcuminoid Content

Total curcuminoid contents of fresh and dried turmeric were in a range of 69.64 – 81.65 and 77.75 – 87.57 mg curcumin/g dry matter, respectively. The influence of drying temperatures on total curcuminoid content is shown in Fig. 3. The results showed that the total curcuminoid contents of dried products tended to be higher compared with the fresh samples. Green *et al.,* (2008) suggested that dried tumeric gave higher levels of curcuminoids (55.5 ± 2.2%) than fresh sample (15.7 ± 0.4%). Tonnensen & Karlsen (1985) did not found a significance in loss of curcumin under 100 °C, but at 125 °C there was %15.25 degradation of the pigment. However, in this study, drying temperature did not show significant effects on total curcuminoid content of the dried samples ($p > 0.05$).

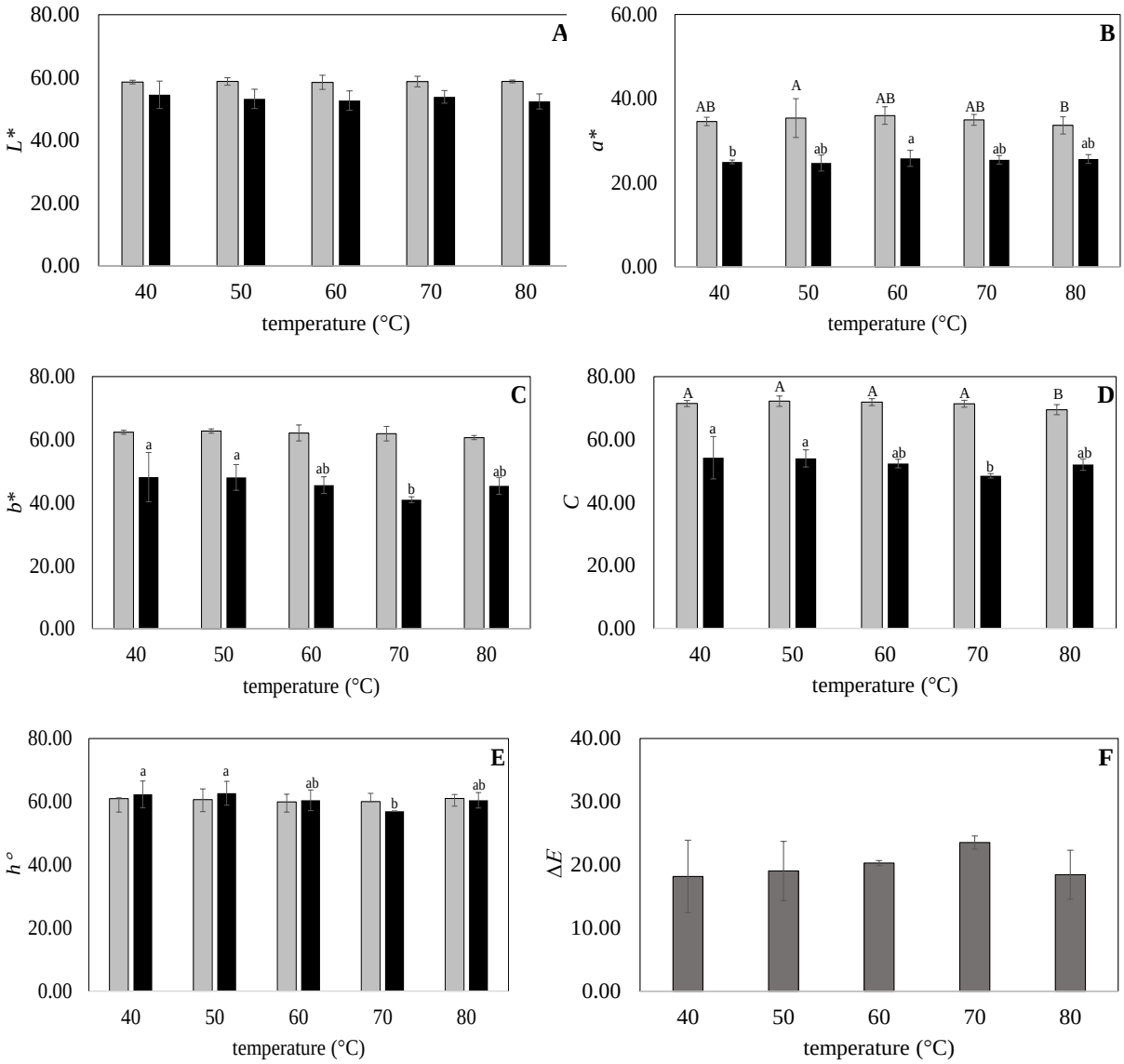


Figure 2: Effects of drying temperature on color of dried turmeric slices including, *L**(A), *a** (B), *b** (C), *chroma* (D), *hue angle* (E) and ΔE (F).

Different capital letters indicated significantly different ($p < 0.05$) of fresh samples from different drying temperatures and different lower-case letters indicated significantly different ($p < 0.05$) of dried samples from different drying temperatures. ■ Fresh turmeric slices ■ Dried turmeric powder

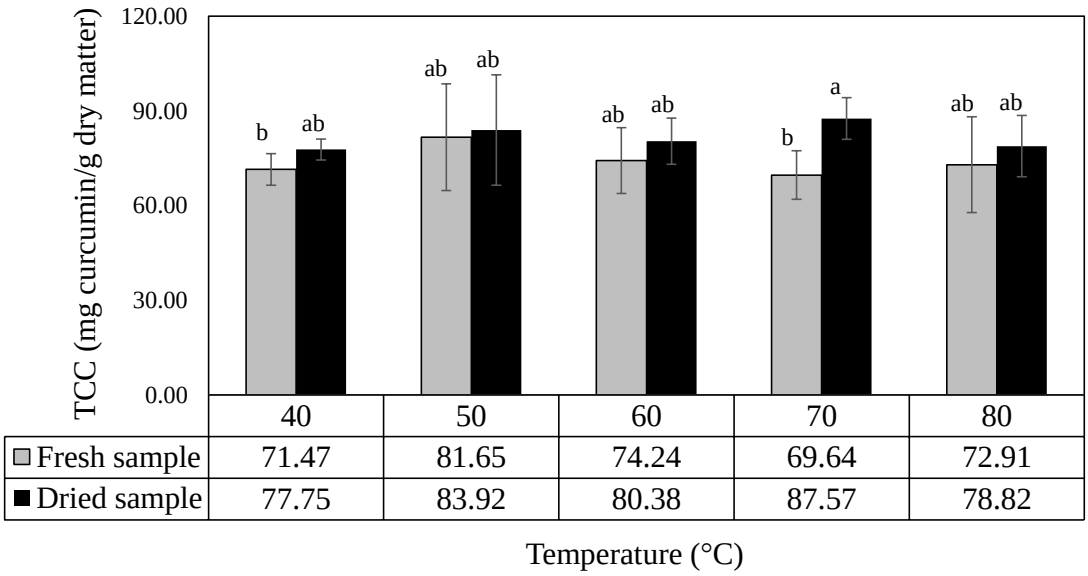


Figure 3: Total curcuminoid contents of fresh and dried turmeric obtained from various temperatures. Bars with different letters are significantly different ($p < 0.05$).

4 CONCLUSION

Drying temperatures did not obviously affect lightness, color components and total color change of dried turmeric slices. Drying at 70 °C resulted in shorter drying time and brighter orange-yellow products. Thus, drying of turmeric slices using a tray dryer can be done at high temperature for accelerating the drying process and reducing the energy consumption without an adverse impact on the color and total curcuminoid contents.

5 ACKNOWLEDGEMENT

We are grateful to the Food Security Center (FSC), University of Hohenheim for the Ph.D. Sandwich Scholarship for N. Komonsing. In addition, we acknowledge Dorothea Hirschbach – Müller and the scientists at the Institute of Agricultural Engineering, Tropics and Subtropics Group for their technical assistances.

REFERENCES

- AOAC. (1999). Official Methods of Analysis of AOAC International. 16th Edition, AOAC, Gaithersburg, MD.
- Argyropoulos, D., Khan, M. T. & Müller, J. (2011). Effect of air temperature and pre-treatment on color changes and texture of dried *Boletus edulis* mushroom. *Drying Technology*, 29, 1890-1900.
- CBI-The Center for the Promotion of Import from Developing Country. Exporting curcuma longa (turmeric) to Europe. (2016). https://www.cbi.eu/market-information/natural-ingredients-health-products/curcuma-longa-turmeric/ Accessed 23 August 2018
- de Lima, M.S., Da Silva, M.A.P., Plácido1, G.R., Cagnin, C., Vieira, N.F., Do Carmo1, R.M., Da Silva1, R.C.F., De Souza Castro1, C.F., Caliari, M. & Silva, R.M. (2016). Physical and chemical characteristics and drying kinetics of turmeric (*Curcuma longa* L.). *African Journal of Agricultural Research*, 12, 28-34.

- de Melo, I.L.P. & de Almida-Muradian, L.B. (2011). Comparison of methodologies for moisture determination on dried bee pollen samples. Ciência e Tecnologia de Alimentos, 31, 194-197.
- de Oliveira, E.F., Tosati, J.V., Tikekar, R.V., Monteiro, A.R. & Nitin, N. (2018). Antimicrobial activity of curcumin in combination with light against *Escherichia coli* O157:H7 and *Listeria innocua*: applications for fresh produce sanitation. *Postharvest Biology and Technology*, 137, 86-94.
- Department of Agricultural Extension. Turmerics. (2016) http://www.agriman.doae.go.th/home/news/year%202016/026_tumeric.pdf. (in Thai) Accessed 23 August 2560.
- Gómez-Estaca, J., Balaguer, M.P., López-Carballo, G., Gavara, R. & Hernández-Muñoz, P. (2017). Improving antioxidant and antimicrobial properties of curcumin by means of encapsulation in gelatin through electrohydrodynamic atomization. *Food Hydrocolloids*, 70, 313-320.
- Green, C.E., Hibbert, S.L., Bailey-Shaw, Y.A., Williams, L.A.D., Mitchell, L, S. & Garraway, E. (2008). Extraction, processing, and storage effects on curcuminoids and oleoresin yields from *Curcuma longa* L. grown in Jamaica. *Journal of Agricultural and Food Chemistry*, 56, 3664-3670.
- Hirun, S., Utama-ang, N. & Roach, P.D. (2014). Turmeric (*Curcuma longa* L.) drying: An optimization approach using microwave-vacuum drying. *Journal of Food Science and Technology*, 51, 2127–2133.
- Karthikeyan, A.K. & Murugavelh, S. (2018). Thin layer drying kinetics and exergy analysis of turmeric (*Curcuma longa*) in a mixed mode forced convection solar tunnel dryer. *Renewable Energy*. 128, 305-312.
- Lakshmi, D.V.N., Muthukumar, P., Layek, A. & Nayak, P.K. (2018). Drying kinetics and quality analysis of black turmeric (*Curcuma caesia)* drying in a mixed mode forced convection solar dryer integrated with thermal energy storage. *Renewable Energy*. 120, 23-34.
- López-Tobar, E., Blanch, G.P., Ruiz del Castillo, M.L. & Sanchez-Cortes, S. (2012). Encapsulation and isomerization of curcumin with cyclodextrins characterized by electronic and vibrational spectroscopy. *Vibrational Spectroscopy*, 62, 292-298.
- Martins, R.M., Pereira, S.V., Siqueira, S., Salomão, W.F. & Freitas, L.A.P. (2013). Curcuminoid content and antioxidant activity in spray dried microparticles containing turmeric extract. *Food Research International*, 50, 657-663.
- Mathew, D. & Hsu, W.L. (2018). Antiviral potential of curcumin. *Journal of Functional Foods*, 40, 692-699.
- Mondal, S., Ghosh, S & Moulik, S.P. (2016). Stability of curcumin in different solvent and solution media: UV-visivle and steady-state fluorescence spectral study. *Journal of Photochemistry and Photobiology*, 158, 212-218.
- Monisha, S., Niveditha, A., Pavithra, D., Chidanand, D. V., Sunil, C. K. & Rawson, A. (2016). Effects of microwave treatment on colour of turmeric (*Curcuma longa* L.). *International Journal of Science, Environment*, 5, 2062-2070.
- Patil, P.B., Parit, S.B., Waifalkar, P.P., Patil, S.P., Dongale, T.D., Sahoo, S.C., Kollu, P., Nimbalkar, M.S., Patil, P.S. & Chougale, A.D. (2018). pH triggered curcumin release and antioxidant activity of curcumin loaded γ-Fe_2O_3 magnetic nanoparticles. *Materials Letters*, 223, 178-181.
- Prathapan, A., Lukhman, M., Arumughan, C. Sundaresan, A. & Raghu, K.G. (2009). Effect of heat treatment on curcuminoid, colour value and total polyphenols of fresh turmeric rhizome. *International Journal of Food Science and Technology*, 44, 1438-1444.
- Siddiqui, N.A. (2015). Evaluation of thermo sensitivity of curcumin and quantification of ferulic acid and vanillin as degradation products by a validated HPTLC method. *Journal of Pharmaceutical Sciences*, 28, 299-305.
- Sikkhamondhol, C., Teanpook, C., Boonbumrung, S. & Chittrepol, S. (2009). Quality of bread with added turmeric (*Curcuma longa*): Powder, essential oil and extracted residues. *Asian Journal of Food and Agro-Industry*, 2, 690–701.

- Strumillo, C. & Kudra, T. (1986). Drying: Principles, applications and design. *Heat and Mass Transfer in Drying Process* (pp. 37-64). New York: Gordon and Breach.
- Homhual, S. Thaicrudedrug: Turmeric. (2010). http://www.thaicrudedrug.com/main.php?action=viewpage&pid=34. (in Thai) Accessed 23 August 2560.
- Tirca, I., Mitran, V., Marascu, V., Brajnicov, S., Ion, V., Stokker-Cherige, F., Popovici, I.A., Cimpean, A., Dinca, V. & Dinescu, M. (2017). *In vitro* testing of curcumin based composites coatings as antitumoral systems against osteosarcoma cells. *Applied Surface Science*, 425, 1040-1051.
- Tonnesen, H.H. & Karlsen, J. (1985). Studies on curcumin and curcuminoids. VI. Kinectics of curcumin degradation in aqueous solution. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 180, 402 – 408.
- Zhang, N., Li, H., Jia, J. & He, M. (2015). Anti-Inflammatory effect of curcumin on mast cell-mediate allergic responses in ovalbumin-induced allergic rhinitis mouse. *Cellular Immunology*, 298, 88-95.

Simultanious Vacuum Falling Film and Rotary Evaporation for Producing Sweet Sorghum Syrup

N. Istianah, S.S.Yuwono, D.Y. Ali*, R.J.A. Aghata

Agricultural Products Technology, Brawijaya University, Jl.Veteran, Malang 65145, Indonesia
*Corresponding Author (dego@ub.ac.id)

ABSTRACT

Simultanious combination of Vacuum Falling Film Evaporator(FFE) at 90 °C and Rotary Evaporator (RE) at 80 °C was used to reduce water content of sweet sorghum and sugarcane juice. Comparison of sweet sorghum within single FFE and simultanious FFE-RE was tested by Least Significant Difference(LSD) or Duncan's Multiple Range Test(DMRT) with 5% confidence interval. Sweet sorghum single FFE concentrate has 44.2°Brix of Total Soluble Solid(TSS) and 30.13 of lightness(L*). They were higher than sugarcane TSS(42.7°Brix) and L*(25.86). Rotary evaporator with the duration of 15, 30, and 45 minutes was used to evaporate the sweet sorghum single FFE concentrate. Overall hedonic test showed the best product is syrup produced by simultanious vacuum falling film evaporator at 90 °C and rotary evaporator at 80°C for 30 minutes. The evidence was the highest overall Hedonic value of 3.34 with the taste parameter value of 2.89; aroma of 3,29; color of 3,75; and thickness of 3.42. It was also accomplished with TSS of 87.53 °Brix; viscosity of 2036.67cP; final L* of 25.83; and reducing sugar of 52.54%.

KEYWORDS: Falling Film Evaporator, Rotary Vacuum Evaporator, Sugar, Sweet Sorghum, Syrup.

1 INTRODUCTION

Sorghum is a kind of plant having wide range of uses. It can potentially be used as raw materials for sugar because it has high content of total sugar (Andrzejewski, 2013). The existing sugar syrup mostly produced by the dillution of sugar crystals. A high amount of energy and cost were spend in this process especially for crystallization, centrifugation, and dillution process. Due to efficiency, evaporation was induced to make sweet sorghum syrup having more total sugar content that was presented in TSS (°Brix) (Lara, 2010). This evaporation contributes to production of sugar that reduces the amount of energy used (Bantacut, 2016) and is waste by existing centrifugation (Bhatnagar, 2016). Vacuum operation was recommended since it can reduce the boiling point of water. Hence, the nutritional losses, browning reaction and taste reduction were minimized. Farahnaky *et al*., (2016) involved a vacuum evaporator to produce date syrup. Falling film evaporation was reported as a good technology for that process (Koodaruth, 2015). At the previous work, it was used to evaporate sugarcane and pinneaple juice with a high rate. In addition, rotary vacuum evaporator has a high performance for food grade product. Therefore, it is necessary to combine falling film and rotary vacuum evaporator simultaniously.

2 METHODS

2.1 Material Preparation

Sweet Sorghum (*Sorghum bicolor (L.) Moench)* harvested in BALITKABI Malang and local sugarcane were used as the material in this research. Both of them were pressed twice using local sugarcane milling machine to produce sweet sorghum and sugarcane juice. Juice was filtered twice using filter cloth 100 mesh and then was centrifuged(WINA Instruments) at 720 rpm for 30 minutes. Zeolite was added into juice before centrifugation with concentration 3% w/w.

2.2 Evaporation

There are two types of evaporation; single FFE and simultanious combination FFE and RE(IKA RV 10). FFE was operated at 90 °C with flow rate of 5 mL/min and RE was operated at 80 °C and rotation speed of 70 rpm within duration of 15, 30, and 45 minutes.

2.3 Data Analysis

Determination of physical properties was measured using color reader (Yuwono and Susanto, 1998), Total Soluble Solid was measured using hand refractometer(Atago N-1E) (Meikapasa and Gusti, 2016), and viscosity(AOAC, 1999) was measured using viscometer (elcometer 2300 RV). Reducing sugar was analized by Nelson-Somogyi (Sudarmadji, 1997) and hedonic test by *Multiple Atribute* with 100 respondens (Soekarto, 2002). Data analysis was conducted using ANOVA (*Analysis of Variance*) with convidence interval 95% and then followed by Duncan's Multiple Range Test(DMRT) with 5% confidence interval.

3. RESULTS AND DISCUSSION

The performance of Falling film evaporator was investigated in use of sweet sorghum and sugarcane juice evaporation. It was examined at temperature of 70, 80 and 90 °C. It shows the different value of TSS. Higher temperature was responsible for the higher TSS of sweet sorghum and sugarcane concentrate. Within the higher temperature steam carried a higher number of heat. The good heat transfer coefficient of FFE(Glover, 2004) helped heat transferred to juice (Adib, 2009). As the result, FFE sucessfully reduced a large amount of water so that TSS increased.

Sweet sorghum has higher potential to be further processed by rotary vacuum evaporator. This was caused by the higher TSS of sweet sorghum concentrate than sugarcane as represented in Table 1. Lo *et al*., (2007) also reported that sugarcane juice has TSS of 15°Brix and the concentrate of 30 °Brix. It was also supported by the higher lightness that was desired on syrup product. Sweet sorghum concentrate has two advantages including high TSS and lighness.

Table 1. Performance of Falling Film Evaporator

Sampel	T(°C)	TSS	Color parameters			Density
		(°Brix)	L	a	b	
Sorghum	70	39±1.57	30.33±0.61	-2.20±0.17	8.57±0.64	1.15±0.01
	80	43.4±0.86	29.87±0.04	-2.47±0.05	7.90±0.08	1.17±0.01
	90	44.2±0.05	30.13±0.09	-2.73±0.22	6.40±0.17	1.18±0.02
Sugarcane	70	30±0.33	25.93±0.25	1.80±0.12	3.80±0.24	1.12±0.01
	80	30.3±0.09	20.70±4.39	-1.86±0.38	1.05±0.08	1.13±0.002
	90	42.7±0.14	25.86±0.29	-1.833±0.39	3.733±0.27	1.16±0.001

Regarding to the high potential of sweet sorghum concentrate, rotary vacuum evaporator can produce sweet sorghum syrup. Operation condition at 80 °C and rotation speed of 70 rpm with duration of 15, 30 and 45 minutes evaluate the performance of rotary vacuum evaporator. It was described by the measurement of TSS, reduction sugar, and linghtness that were showed in Table 2.

Table 2. Performance of Rotary Vacuum Evaporator

Time (min)	TSS (%)	DMRT 5%	Gula Reduksi (%)	DMRT 5%	Lightness	DMRT 5%
15	50.33 ± 1.52	1.726	48.22 ± 2.43	3.321	29.55 ± 0.82	0.74
30	87.53 ± 0.50	1.767	52.54 ± 1.45	3.353	25.82 ± 0.57	0.80
45	96.33 ± 1.15	-	53.24 ± 3.02	3.379	24.99 ± 0.41	0.85

Table 2. showed that further evaporation of sweet sorghum concentrate using rotary vacuum evaporator could increase the value of TSS significantly. It was also followed by the higher value of

reducing sugar. TSS value that was higher than reducing sugar means there was sucrose inside of syrup. TSS value increased according to reducing sugar since there was soluble sucrose (Crestani, 2017) and reducing sugar in sweet sorghum syrup recorded in TSS.

Fig.1 showed that longer duration of evaporation caused the higher TSS and reducing sugar. The increase of TSS was higher than reducing sugar within 15 to 45 minutes of evaporation by RE. It showed sweet sorghum syrup has high content of sucrose causing the sweet taste. That was a great result since it has a role in making product with a good taste.

The slight increase of reducing sugar was caused by the minimum coversion of sucrose to reducing sugar. Vacuum operation of simultanious FFE-RE could reduce the converting sugar that was mainly promoted by hydrolisis reaction(Gehlawat, 2001) in a high temperature process. Panpae (2008) also reported that higher temperature made it aesier to break sucrose into invert sugar. This invert sugar has disadvatages in food application because it causes the browning reaction. Therefore, simultanious FFE-RE was capable to produce a safe sweet sorghum syrup.

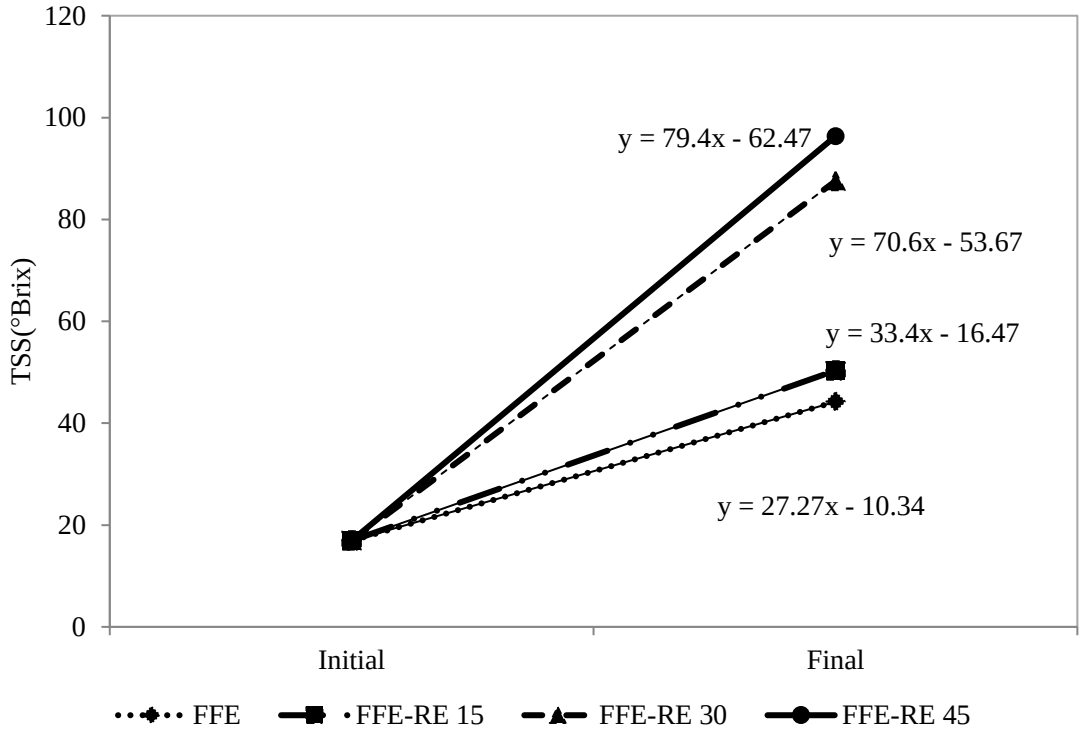


Figure 1. Total Soluble Solid of sweet sorghum syrup

Within the increase of TSS and reducing sugar, the lightness of sweet sorghum syrup lightly decreased. It was the result of water losses by evaporation. The less water content and more solid content changed sweet sorghum syrup color to be darker. The longer exposure of evaporation heat to the syrup increased the darkness of syrup.

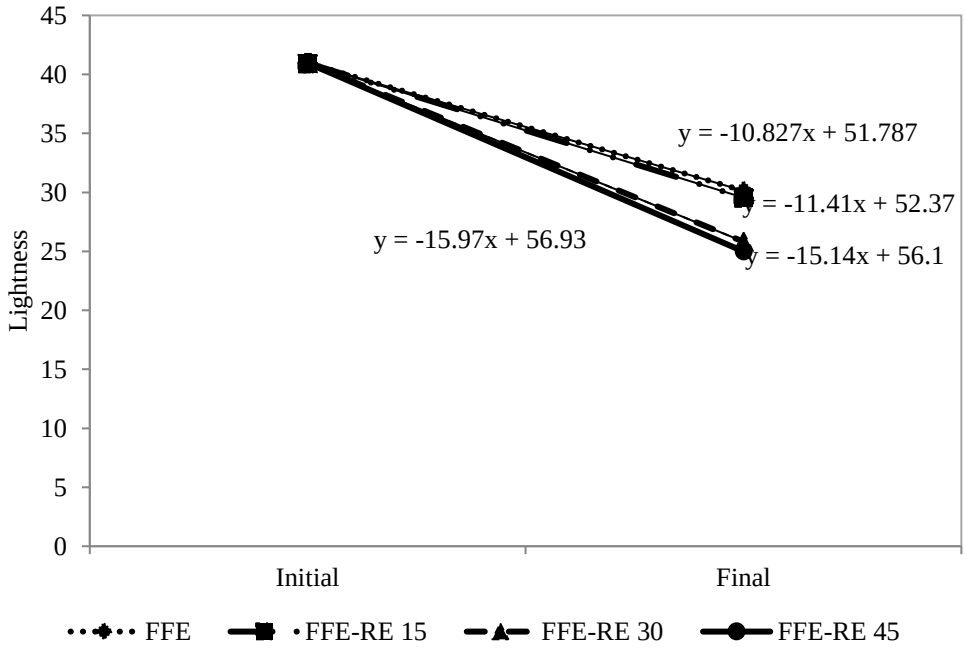


Figure 2. Lightness of of sweet sorghum syrup

The increase of TSS value slightly affected the lightness of sweet sorghum syrup. Fig 1. and Fig 2. informed that TSS changes of sweet sorghum syrup was higher than lightness changes. It has lower gradient (15.97) than gradient of TSS changes (79.4). Lightness reduction means the production of dark color that was mainly caused by the browning reaction. It is a kind of termal behavior of sugar(Lu, *et al.,* 2017). It means the decrease of lightness was not dominately influenced by sugar content. The centrifugation of raw juice (Caroline, 2012) and addition of zeolite (Istianah, 2017) also helped maintain the concentrate lightness.

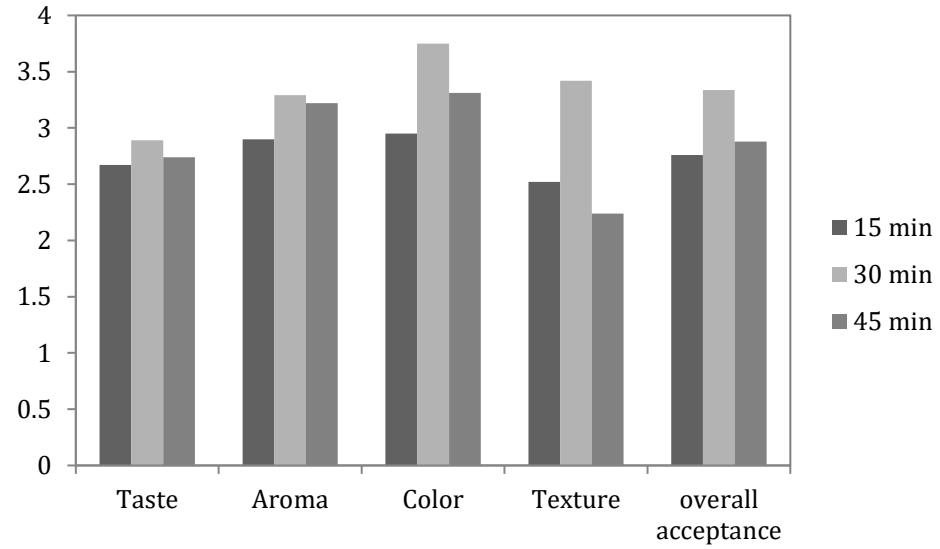


Figure 3. Hedonic value of sweet sorghum syrup

Simultanious evaporation of sweet sorghum juice using falling film evaporator and rotary vacuum evaporator successfully produced sweet sorghum syrup. Hedonic test showed the most preferable product. The highest hedonic value is the color of product conducted by simultanious evaporation of falling film evaporator at 90 °C and rotary vacuum film evaporator at 80 °C within 30minutes. It was also proven by the highest value of overal acceptance and other parameters such as taste, aroma and texture(viscosity).

Among all hedonic parameters, color has the highest value. It was followed by texture, aroma, overal acceptance and taste. This value proved that simultanious evaporation provided a good color of light product. It was because of less browning reaction during evaporation. Simultanious evaporation by 30 minutes also gives a good water content of product.

On the other hand, taste has the lowest value. It was caused by the origin presence of clorophyl. It was a kind of antioxidant needed to increase health. However, better taste was a priority for consumer. Hence, sweet sorghum syrup needs to be developed to improve the taste value.

4 CONCLUSSION

Simultanious evaporation using falling film evaporator at 90 °C and rotary vacuum film evaporator at 80 °C within 30 minutes can produce sweet soghum syrup having high TSS value of 87.53°Brix, reducing sugar of 52.54%, viscosity of 2036.67cP; and final L* of 25.83. It has the highest overall Hedonic value of 3.34 with taste parameter value of 2.89; aroma of 3.29; color of 3.75; and thickness of 3.42.

5 ACKNOWLEDGMENT

This work was supported by Lembaga Penelitian dan Pengabdian Masyarakat(LPPM) and Laboratory of Food Processing and Engineering of Brawijaya University.

REFFERENCES

- Adib, Tarif Ali., Bertrand Heyd and Jean Vasseur. (2009). Experimental Results and Modeling of Boiling Heat Transfer Coefficients in Falling Film Evaporator Usable for Evaporator Design. *Chemical Engineering and Processing: Process Intensification* 48(4), 961-968.
- Andrzejewski, Brett., Gillian Eggleston. (2013). Pilot plant clarification of sweet sorghum juice and evaporation of raw and clarified juices. *Industrial Crops and Products* 49, 648– 658.
- AOAC. (1999). Official Method of Analysis. 16th edition. Association of Official Analytical Chemist International : USA
- Bantacut, Tajuddin and Destiara Novitasari. (2016). Energy and water self-sufficiency assessment of the white sugar production process in Indonesia using a complex mass balance model. *Journal of Cleaner Production 126*, 478-492.
- Bhatnagar, Amit., Kavindra Kumar Kesari and Narasinha J. Shurpali. (2016). Multidisciplinary Approaches to Handling Wastes in Sugar Industries. *Water Air and Soil Pollution* 227(1), 1-30.
- Thai, C. C. D., H. Bakir, and W. O. S. Doherty. 2012. Insights to the Clarification of Sugar Cane Juice Expressed from Sugar Cane Stalk and Trash. *J. Agric. Food Chem*., 60 (11), 2916–2923.
- Farahnaky,A., M. Mardani, G. Mesbahi, and Mohammad-Taghi Golmakani. (2016). Some Physicochemical Properties of Date Syrup, Concentrate, and Liquid Sugar in Comparison with Sucrose Solutions. *Journal of Agricultural Science and Technology* 18(3):657-668.
- Glover, William B. (2004). Selecting Evaporators for Process Applications. Chemical Engineering Process Magazine, 26-33.
- Istianah, Nur; Dego Yusa Ali, and Sudarminto Setyo Yuwono. (2017, oktober). Adsorption Separation and Multistage Evaporation using Falling Film Evaporator(FFE) to Produce Sugar Syrup. Proceedings of The 1st International Conference on Green Agro-industry and Bioeconomy. Malang.
- Koodaruth, Abdel. (2015). Use of Falling Thin Film Evaporator for Increasing Cogenerated Electricity in Cane Flexi-factory in Mauritius. Energy Procedia 75, 1021-1026.
- Lara, Nelly and Alejandra Clavijo. (2010). Study of The Sugar Cane Juice Concentration during Production of Granulated Panela by Direct Boiling in Open Systems at Environment Conditions. International Conference on Food Innovation 25-29 October, 1-4.

- Lo, W.M., L.S.T. Chua, Abbas Fadhl Al-Kharkhi and M.E. Azhar. (2007). Evaluation of freeze-concentrated sugar-cane juice. *Trop. Agric. and Fd. Sc*. 35(1), 121–129.
- Lu, Yingshuang, LeiLei Yin, Danielle L. Gray, Leonard C. Thomas, and Shelly J. Schmidt. (2017). Impact of sucrose crystal composition and chemistry on its thermal behavior. *Journal of Food Engineering* 214, 193-208.
- Meikapasa, N.W. P and I Gusti N.O.S. (2016). Characteristics of Total Dissolved Solids (TSS), Lycopene Stability and Vitamin C of Tomato Sauce. *Ganec Swaras Journal* 10(3), 81-86.
- Panpae, Kornvalai., Wasna Jaturonrusmee, Withawat Mingvanish, Chantana Nuntiwattanawong, Surapon Chunwiset, Kittisak Santudrob and Siriphan Triphanpitak. (2008). Minimization of Sucrose Losses in Sugar Industry by PH and Temperature Optimization. *The Malaysian Journal of Analytical Sciences*, 12,(3),513 – 519.
- Sudarmadji, S. Haryono. (1997). Prosedur Analisa Untuk Bahan Makanan dan Pertanian. Liberty : Yogyakarta
- Soekarto. (2002). Penilaian Organoleptik Untuk Industri Pangan dan Hasil Pertanian. IPB : Bogor.
- Yuwono, S. dan Susanto, T. (1998). Pengujian Sifat Fisik Pangan. Fakultas Teknologi Pertanian, UB : Malang.

Detection of 3-monochloropropane-1,2-diol (3-MCPD) Using Molecularly Imprinted Poly(3-aminophenylboronic acid)

Niroshan Chinthaka Gunawardena*, Loc Thai Nguyen

Asian Institute of Technology, Klong Luang, Thailand
*Corresponding Author (niroshan170@gmail.com)

ABSTRACT

A screen printed carbon electrode (SPCE) modified with multiwalled carbon nanotubes (MWCNT), gold nanoparticles (AuNP) and molecularly imprinted (MIP) poly (3-aminophenylboronic acid) was developed as the sensing platform to detect 3-monochloropropane-1,2-diol (3-MCPD). In this study, 3-aminophenylboronic acid (3-APBA) was employed due to its affinity towards cis-diol compounds through reversible ester formation. Morphological and electrochemical characterizations of the sensor were done using FE-SEM/EDX and potentiometry, respectively. In comparison to the non-imprinted sensor, the MIP sensor demonstrated a statistically significant ($p < .05$) response towards 3-MCPD in the range 600 to 1500 µM. The response of the imprinted sensor was two times higher than the response of the non-imprinted sensor. Fabrication and measurement conditions were optimized for the concentration of CNT dispersion, AuNP electrodeposition cycles and electropolymerization cycles of the MIP. The effect of the pH of phosphate buffer solution on measurement was also assessed. The optimized sensor showed a linear range at 300 to 1500 µM and the sensitivity was 0.011 mV/µM. The LOD and LOQ of the sensor were determined to be 192.53 µM and 583.42 µM, respectively. The sensor exhibited rather a good selectivity against 1,3-DCP, 1,2-propanediol, glycerol, and D-glucose. The potentiometric response of the sensor was moderately reproducible with an RSD of <15%. High stability of the sensor was confirmed by the non-significant change ($p >.05$) in response over 20 days of storage at room temperature. The developed sensor can be further developed for sensitive and selective detection of 3-MCPD in real food samples.

KEYWORDS: 3-MCPD, Aminophenylboronic Acid, SPCE, Molecular Imprinting

1 INTRODUCTION

Chloropropanols is a group of process contaminants consisting of at least 5 major compounds. Among them, 3-chloropropane-1,2-diol (3-MCPD) and 1,3-dichloropropane-2-ol (1,3-DCP) are mostly considered as they are process induced contaminants found in food. 3-MCPD is produced as a repercussion of the production of acid-hydrolysed vegetable protein (acid-HVP). It is formed from acid-HVP through the reaction of HCl with triacylglycerols, phospholipids, and glycerol from the residual vegetable oil. Acid-HPV is a frequent constituent in soups, prepared meals, savoury snacks, gravy mixes and stock cubes (Codex, 2001). 3-MCPD was first found in hydrolysed vegetable protein, soy sauce, and similar foods in 1978 by Velíšek *et al*., Consumption of medium to high 3-MCPD amount during a short time period is related to kidney and reproductive organ failure (Lee & Khor, 2015). The International Agency for Research on Cancer (IARC) has graded 3-MCPD to be a "Group 2B carcinogen" indicating its possibility to be carcinogenic to humans (IARC, 2017). The Office of Environmental Health Hazard Assessment (OEHHA) has characterized 3-MCPD as "Proposition 65 (Prop. 65)", referring it as a substance capable of causing cancer, birth deformities, and other reproductive impairment (OEHHA, 2010). Presence of 3-MCPD in food is regulated in European Union (EU) legislation EC 1881/2006 with a maximum level of 0.02 mg/kg in food and a tolerable daily intake (TDI) of 2 µg/kg b.w. (Commission, 2006). US Food and Drug Administration (FDA) has instituted a maximum level of 1 mg/kg. Similarly, various limits of 3-MCPD have been imposed at country and organization levels. Prevailing analytical methods for the quantification of 3-MCPD involve extensive sample preparation coupled with GC-MS which is time consuming, expensive and require special expertise. Therefore, it is essential to develop real-time analytical devices with high sensitivity and selectivity at an affordable price.

Generally, electrochemical sensors react with a target analyte to bring about a change in electrical signal (current/potential/conductivity) relative to the analyte concentration. Electrochemical sensors with nanomaterial incorporation have the capability of attaining improved sensitivity, selectivity and response time, to meet the requirement of contaminant sensing in complicated food matrices (Zeng *et al.,* 2016). Screen printed electrodes (SPE) have received a considerable attention as a low cost electrochemical sensing approach with the ability of different surface modifications for a wide range of analytical applications. Molecular imprinting is a technology for the institution of recognition properties in synthetic polymers. In the recent times, molecularly imprinted polymers (MIPs) have become a principal interest for the development of chemical and biological sensors due to their considerable advantages in comparison to natural receptors like enzymes, antibodies, etc. The MIP-based sensors combining molecular imprinting with electrochemical sensors have a superior ability to selectively sense a target of interest. MIPs used in these sensors serve as specific recognition elements. The incorporation of molecular imprinting technology has further enhanced the use of screen printed electrodes as a highly selective sensing element.

The complexation reaction of boric and boronic acid compounds with saccharides was discovered in 1954 (Zhong *et al.,* 2015). Since then, a number of diol sensing strategies have employed many boronic acid derivatives. Amongst them, development of electrochemical and optical sensors based on this mechanism has attracted much attention (Zhong *et al.,* 2015). Polymerizable boronic acid derivatives have also been made as monomers to form MIP. This boronic acid based MIPs have the ability to distinctly distinguish template molecule from its cis-diol analogues let alone their selectivity towards cis-diol compounds from general compounds. Owing to eminent operational stability and polymerizable property (Zhong *et al.,* 2015), phenylboronic acid (PBA) has been the extensively used boronic acid derivative. It is known that boronic acids bind with high affinity with diol moiety containing compounds through "reversible ester formation". This rigid binding has enabled boronic acid to be used as the recognition moiety in the building of sensors (Springsteen & Wang, 2002). Nevertheless, the conditions that foster solid binding between the diol and the boronic acid are yet to be fully comprehended.

Herein we report the development of a novel and selective electrochemical sensor based on MWCNT/AuNP coated molecularly imprinted poly(3-aminophenylboronic acid) for the detection on 3-MCPD. The complexation reaction between boronic acid-derived conducting polymer (3-APBA) and diol functionality of 3-MCPD enhanced through molecular imprinting was utilized as the recognition moiety which was modified on a screen printed carbon electrode. The potentiometric technique was employed for detection inside a stirred phosphate buffer solution at pH 7.4. The parameters of the sensing platform were optimized and the analytical characteristics were evaluated under the optimized conditions. Development of this electrochemical sensor will open a new avenue for the utilization of 3-APBA in food safety applications.

2 EXPERIMENTAL

2.1 Materials and Equipment

3-aminophenylboronic acid monohydrate ($C_6H_8BNO_2 \bullet H_2O$, 97%), (±)-3-chloro-1,2-propanediol ($C_3H_7ClO_2$, 98%), 1,3-dichloro-2-propanol ($C_3H_6Cl_2O$, 98+%) and 1,2-propanediol ($C_3H_8O_2$, 99.5%) were purchased from Alfa Aesar (USA). Gold (III) chloride solution ($HAuCl_4$, 99.99% trace metals basis, 30 wt. % in dilute HCl) was purchased from Sigma Aldrich (USA). Glycerol ($C_3H_8O_3$, 99%), D-glucose ($C_6H_{12}O_6$, >95%), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$, 99%) and sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$, 99%) were from Ajax Finechem (Australia). Carboxyl functionalized multi-walled carbon nanotube (MWCNT-COOH) dispersion was kindly provided by Center for High Technology Development – VAST (Vietnam). All chemicals were analytical reagent grade and were used without further purification. Double distilled water was used for chemical preparation. Phosphate buffer solution (PBS) was prepared by mixing monobasic and dibasic stock solutions in different proportions to get the desired pH. SPCE having carbon working (ø = 3 mm), carbon counter and Ag/AgCl reference electrodes were obtained from Quasense Co. Ltd. (Thailand). Electrochemical measurements were conducted using USB powered mini potentiostat DY2100B (Digi-Ivy Inc, USA) interfaced to a personal notebook computer. The surface morphology of the electrode at

different modification stages was characterized using field emission scanning electron microscope (S-4800, Hitachi, Japan) operating at 5.0 kV and a working distance of 3.8 mm. Elemental composition of the modified electrode was analysed using the same scanning electron microscope (SEM) with energy dispersive x-ray spectroscopy (EDX) technique.

2.2 Electrode Modification

A stock solution of MWCNT-COOH having a concentration of 0.3 g/mL was sonicated for one hour in the ultrasonic cleaner and diluted with deionized water to a final concentration of 0.3 mg/mL. 6 µL of the diluted CNT dispersion was drop-casted on the working electrode surface of SPCEs followed by drying at room temperature until absolute evaporation (Lamas-Ardisana, *et al*., 2008; Dago *et al*., 2015). The CNT modified electrode was termed MWCNT/SPCE. The electrode was further modified with AuNPs. 150 µL of 5 mM $HAuCl_4$ solution in 0.1 M KCl was placed on the MWCNT/SPCE and electrodeposition was carried out by 15 cyclic voltammetric scanning at the potential range of 0.0 V to 1.1 V at a scan rate of 50 mV/s for 15 cycles (Do *et al.*, 2014; Viet & Takamura, 2016). The solution on the AuNP modified electrode (MWCNT/AuNP/SPCE) was blotted with a paper towel and five cyclic voltammetric scannings from 0.0 V to +0.4 V in deionized water was carried out at a scan rate of 50 mV/s to rinse the electrode.

The preparation of molecularly imprinted electrode (MWCNT/AuNP/MIP/SPCE) was carried out in in 0.5 M HCl containing 20 mM 3-APBA, 100 mM NaF and 50 mM 3-MCPD template (Zhou *et al.*, 2014). 150 µL of the polymer solution was placed on the SPCE surface and electropolymerization was carried out by cyclic voltammetric scanning from 0.1 V to +1.2 for 5 cycles at a scan rate of 100 mV/s (Sun *et al*., 2014; Badhulika & Mulchandani, 2015; Zhong *et al.*, 2015; Wang *et al.*, 2016; *Wu et al.*, 2017). Afterwards, the solution was blotted with a paper towel and the electrode washed with deionized water. This was followed by template removal using cyclic voltammetric scanning from 0.0 V to +0.6 V in 0.1 M H_2SO_4 solution (Zhong *et al.*, 2015; Wang *et al.*, 2016) for 15 cycles at a scan rate of 100 mV/s. Further cyclic voltammetric scanning in deionized water at the same condition for 5 cycles was done to remove any traces of acid on the electrode surface. The final modified electrode was dried at room temperature before being used for any experiment. Non-imprinted electrode (MWCNT/AuNP/NIP/SPCE) was fabricated following the same procedure without the inclusion of 3-MCPD template in the polymer solution.

2.3 Electrochemical Measurements

Electrochemical measurements were carried out using the three-electrode system with the MWCNT/AuNP/MIP/SPCE. Potentiometric method (sample time = 0.1 sec, constant current = 1 µA) was employed for measurements. Unless otherwise mentioned, all potentiometric experiments were conducted in 185 mL of 0.1 M stirred PBS at pH 7.4. Before each test sample, a blank sample was conducted in the same buffer solution for the whole time period of the test sample in order to condition the freshly prepared electrode. The electrode potential was settled in the buffer solution for 200 seconds before the addition of 3-MCPD. Change in potential was recorded as a function of time throughout the experiment run time. ΔE was determined as the potential value of 0.1 M buffer with 3-MCPD, after subtracting the background potential of 0.1 M buffer without 3-MCPD. All electrochemical measurements were conducted in triplicate at the room temperature (28 ± 2 °C).

3 RESULTS AND DISCUSSION

3.1 Electrode Modification and Characterization

AuNPs were electrodeposited on the MWCNT/SPCE. Figure 1 shows the first and tenth cycles of AuNP deposition. As observed through an increase in current with a higher number of deposition cycles, the conductivity of the electrode increased. The peak observed around 0.3 V represent the Au^{3+} reduction peak as reported before (Do *et al.*, 2014; Devkota *et al*., 2018). Increase in conductivity and appearance of reduction peak confirms the successful electrodeposition of AuNP on MWCNT/SPCE. The current crossover between the forward scan and the backward scan occurred around 0.55 V. This

is in close agreement with Hezard *et al.*, (2012) who have reported it to be 0.62 V for glassy carbon electrode. The shifting of the reduction peak towards negative potential with the increment of scan cycles indicated a progressive weakening of Au deposition (Hezard *et al.*, 2012) possibly due to the limited Au availability in the $HAuCl_4$ solution placed on the electrode.

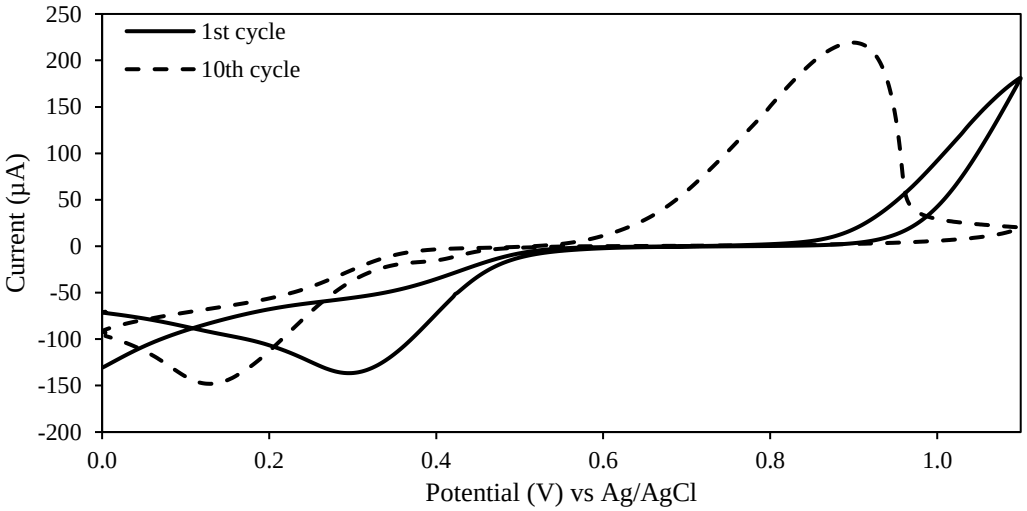


Figure 1: Cyclic voltammograms of the first and tenth cycle obtained during the electrodeposition of AuNP on MWCNT/SPCE

Electropolymerization of 3-APBA was carried out on the MWCNT/AuNP/SPCE both in the presence and absence of the 3-MCPD template molecules. With the increasing number of scan cycles, the current was decreased for both MIP and NIP processes (Figure 2) and continual cycling ensued in the deposition of a redox active film of poly(3-APBA) on the electrode surface (Shoji & Freund, 2001; Deore & Freund, 2003). The less electroactive nature of 3-MCPD could possibly have caused the similarity in the change of current density for both MIP and NIP film growth. Only an oxidation peak was observed for both MIP and NIP in the first scan. However, both oxidation and reduction peaks were seen on subsequent scans for both MIP and NIP. Oxidation and reduction peaks of both imprinted and non-imprinted polymer were closely located. The oxidation peak of 3-aminophenlyboronic acid occurred around 0.35 V and the reduction peak occurred around 0.42 V which were comparable with previous work (Shoji & Freund, 2002; Li *et al*., 2014; Zhou *et al*., 2014). The observed reduction peak could be ascribed to the transition of the polyaniline backbone from the leucoemeraldine salt to the emeraldine salt (Shoji & Freund, 2001; Shoji & Freund, 2002).

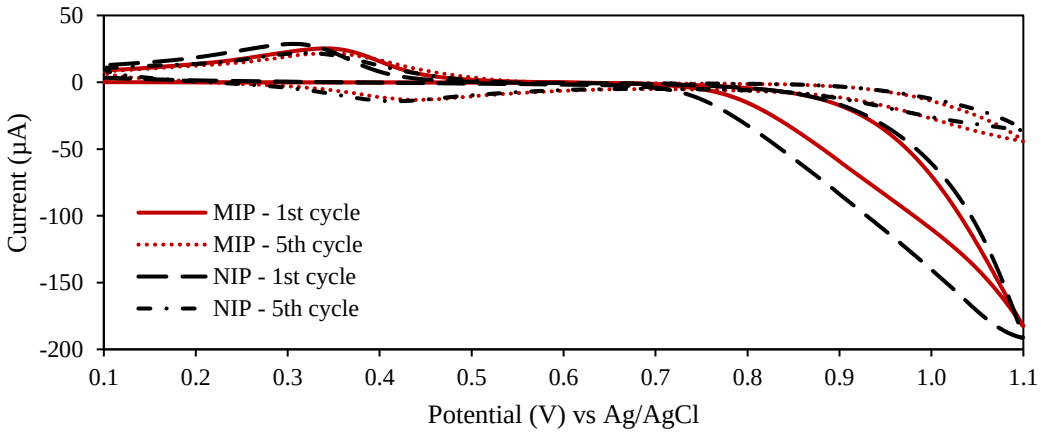


Figure 2: Cyclic voltammograms of the first and the fifth cycle obtained during electropolymerization of 3-APBA in the presence and absence of 3-MCPD template

Surface morphologies of the electrodes at different modification stages as observed by SEM are shown in Figure 3. The bare surface of the SPCE could be characterized by its typical rough nature with microporous structures (Devkota *et al.*, 2018). Modification with MWCNT was indicated by the presence of a characteristic spaghetti-like structure of CNTs (Muhammad *et al.*, 2018). Few bright spots observed on (**C**) showed a scarce distribution of AuNPs which was 0.22 atomic % of Au on the final electrode according to the EDX analysis. Electropolymerization of 3-APBA caused a further change in the surface morphology of the final MIP electrode.

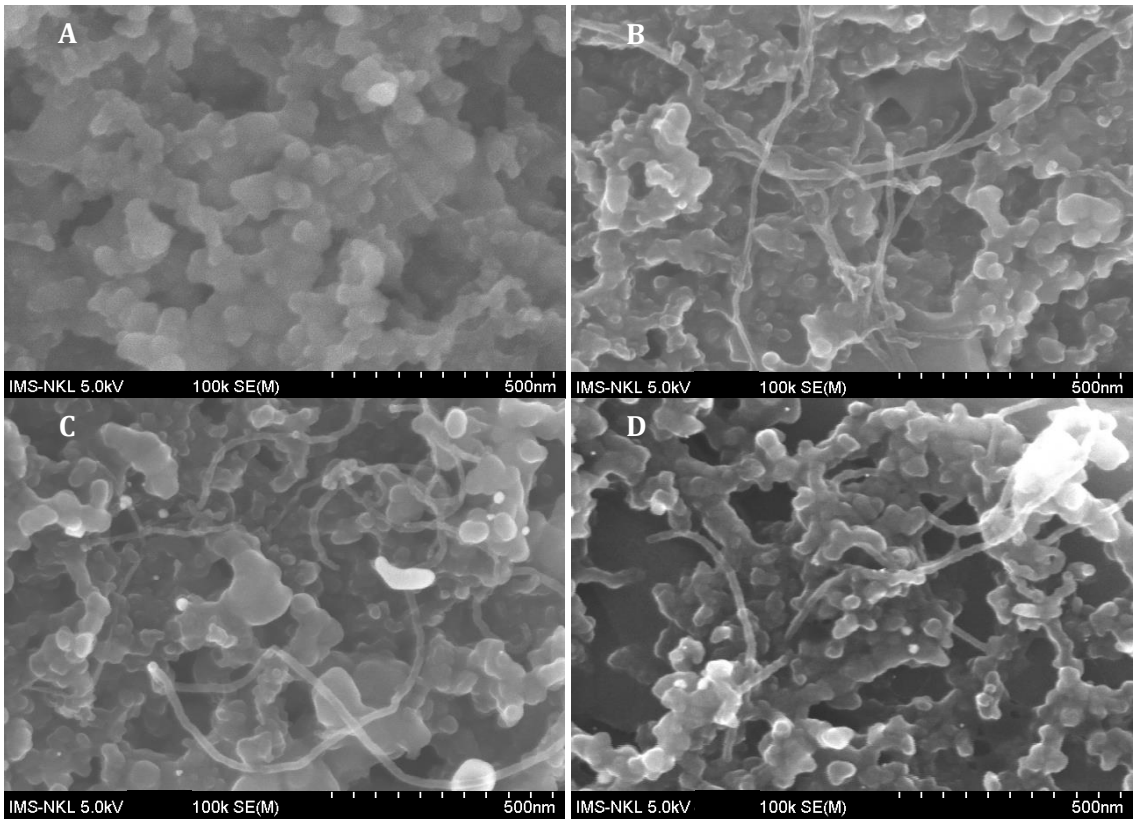


Figure 3: SEM images of (**A**) Bare SPCE, (**B**) MWCNT/SPCE, (**C**) MWCNT/AuNP/SPCE and (**D**) MWCNT/AuNP/MIP/SPCE

Electrochemical characterization of the developed sensor was investigated by assessing the potentiometric response upon the addition of 3-MCPD in the range 300 to 1500 µM (Figure 4.**A**). Although a response was recorded for SPCE, CNT/AuNP/NIP/SPCE and CNT/AuNP/MIP/SPCE, the response shown by the bare SPCE was associated with high noise. On the other hand, both NIP and MIP electrodes depicted a clear response curve. The potential change of imprinted sensor was higher than that of the non-imprinted sensor at all concentration levels of the linear range (Figure 4.**B**). Statistical analysis further rendered that the potential change measured by MIP sensor was significantly higher than that of NIP sensor (p <.05) for the 3-MCPD concentration range of 600 to 1500 µM. This proved the specific binding ability of the MIP sensor towards 3-MCPD. The response shown by the NIP sensor was due to the non-specific surface binding of 3-MCPD with the polymer through boronic acid-diol recognition.

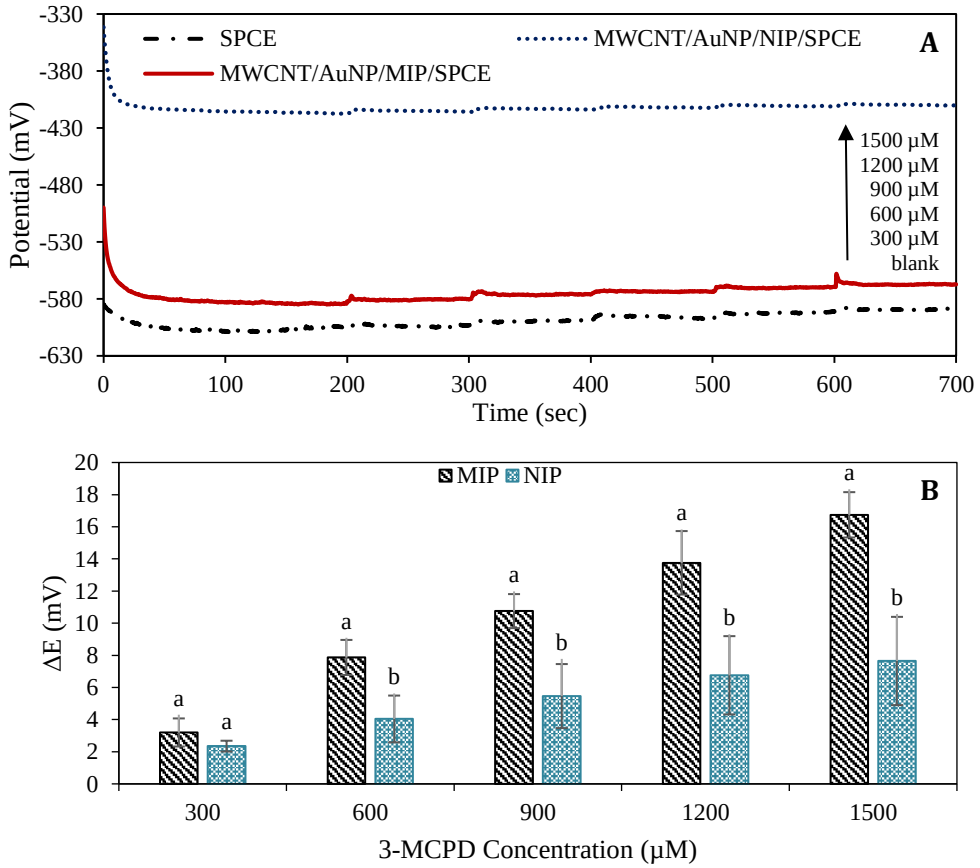


Figure 4: (**A**) Potentiometric response curve upon the addition of 3-MCPD. (**B**) Comparison of potentiometric response on MIP and NIP sensor. (Error bars indicate S.D & letters indicate significant difference at, $p < .05$)

3.2 Optimization of the Conditions

The cycle number of 3-APBA electropolymerization was first optimized in order to find out the desirable polymer coating for an effective 3-MCPD response. It is reported that the number of electropolymerization cycles is crucial for the formation of MIP film as it directly influences the thickness of the polymer grown on the electrode surface (Rezaei et al., 2014). The optimization was done for 2, 5, 10, 15, 20 and 25 cycles. Potentiometric response upon the sequential addition of 3-MCPD in the concentration range of 300 to 1500 µM was recorded. The potential change for 300 µM was separately plotted (Figure 5.**A**) at each cycle number. Accordingly, the highest response potential was measured for 10 cycles (5.63 ± 1.63 mV) followed by 2 (4.59 ± 0.94 mV) and 5 (4.01 ± 0.60 mV) cycles. However, the standard deviation for the response of 10 cycles was high. On the other hand, although response deviation for 2 cycles was lower than 10 cycles and slightly higher than 5 cycles, it was not considered since 2 polymerization cycles would mean a very thin film growth on the electrode. Therefore, 5 electropolymerization cycles were selected as the optimized condition.

Most of the previous studies have not optimized the concentration of MWCNT for the electrode modification. Nevertheless, the concentration of MWCNT was optimized in this study for different concentration levels from 0.15 mg/mL to 1 mg/mL (based on literature) in order to determine the best suiting amount for electrode modification. Thus modified electrodes followed by electropolymerization were used to record the potentiometric response upon the sequential addition of 3-MCPD in the concentration range of 300 to 1500 µM. Comparison of the potential change for 300 µM (Figure 5.**B**) depicted that the highest response (4.76 ± 2.40 mV) was measured for 0.15 mg/mL followed by 0.3 mg/mL (4.28 ± 1.57 mV). The lowest response (2.45 ± 1.39 mV) was recorded for 0.5 mg/mL. Considering the standard deviation and the noise of the response, 0.3 mg/mL CNT concentration was chosen as the optimized level for the modification. Overall, there was no clear trend observed.

Therefore, it is necessary to further investigate this observation within this range at smaller intervals while considering the binding nature of CNT with 3-APBA polymer.

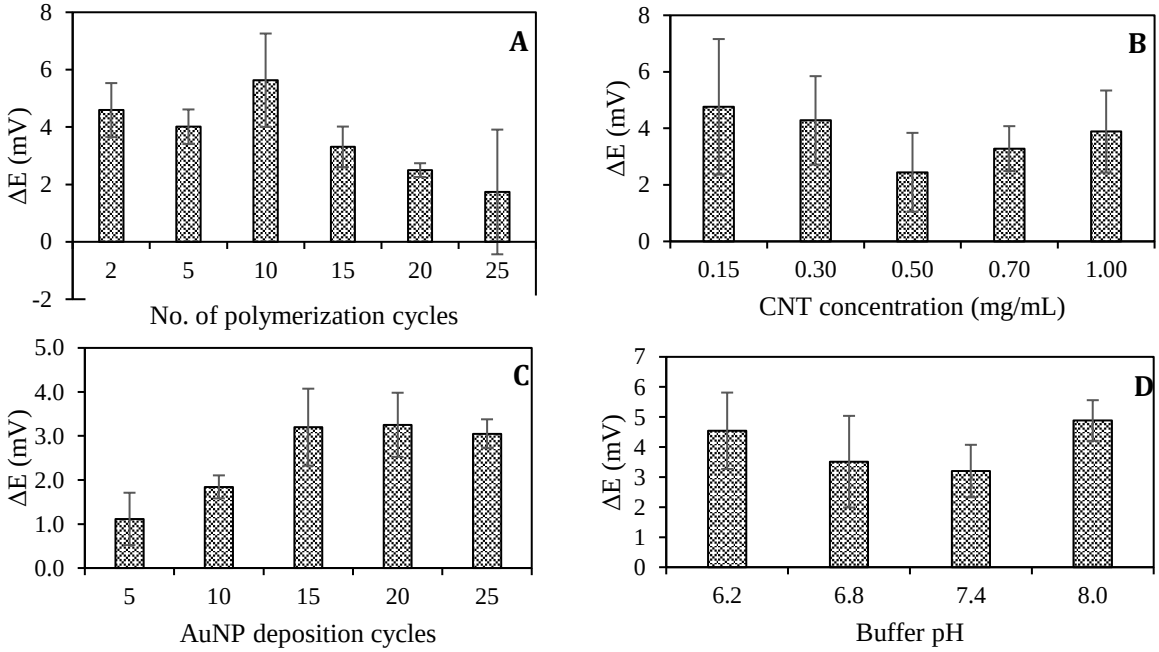


Figure 5: Potentiometric response upon the addition of 300 µM 3-MCPD for different (**A**) electropolymerization cycles, (**B**) MWCNT concentration, (**C**) AuNP electrodeposition cycles and (**D**) pH of 0.1 M PBS. (Error bars indicate S.D)

Electrodeposition parameters such as scan rate and scan cycles can be manipulated to control the growth of metal nanoparticles. In this study, electrodeposition cycles of AuNP on MWCNT/SPCE was optimized for 5, 10, 15, 20 and 25 cycles. Potentiometric response for the thus modified electrode followed by 5 electropolymerization cycles, was recorded upon the sequential addition of 3-MCPD in the concentration range of 300 to 1500 µM. When comparing the response for 300 µM (Figure 5.**C**), the highest potential change was measured for 20 cycles (3.25 ± 0.73 mV) which was closely followed by 15 cycles (3.20 ± 0.87 mV) and the lowest response was recorded for 5 cycles (1.12 ± 0.59 mV). Overall, the potentiometric response increased from 5 cycles to 15 cycles portraying the conductivity increase due to AuNP and the response was stable from 15 to 20 cycles followed by a slight decrease at 25 cycles suggesting that the beneficial effect of AuNP is dependent on the number of deposition cycles. Ultimately 15 cycles of AuNP electrodeposition was selected as the optimum condition.

A phosphate buffer of pH 7.4 was initially selected in this study, following most of the previous work dealing with boronic acid based sensors. However, the potentiometric response for MWCNT/AuNP/MIP/SPCE sensor upon the sequential addition of 3-MCPD in the concentration range of 300 to 1500 µM in 0.1 M PBS having different pH values of 6.2, 6.8, 7.4 and 8.0 was recorded in order to assess the change in response at varying buffer pH. As seen in Figure 5.**D**, the highest response (4.88 ± 0.68 mV) for 300 µM was measured for the buffer at pH 8.0 followed by the buffer at pH 6.2 (4.54 ± 1.27 mV). The lowest response (3.20 ± 0.87 mV) was obtained for the pH 7.4 PBS. Notwithstanding, better response curve with low noise was obtained at pH 7.4. It is known that the complexation reaction of diol compound with aromatic boronic acid is pH dependent and the association is favoured with increasing pH albeit the mechanism is not fully understood. It is possible that the optimum pH condition for the boronate ester formation is above the pK_a of the boronic acid species (in this case 3-APBA, pK_a=8.9). However, the pK_a of the diol compound (3-MCPD, pK_a=13.6) has also an influence on the optimum pH (Yan *et al.*, 2004; Van *et al.*, 1984; Sienkiewicz & Roberts, 1980).

3.3 Performance of the Modified Electrode

Potentiometric response upon the sequential addition of 3-MCPD (50 µM to 300 µM & 300 µM to 1500 µM) was recorded for the optimized MWCNT/AuNP/MIP/SPCE in pH 7.4 PBS. ΔE was plotted against the concentration and a linear regression line was fitted for both ranges. The potential change increased linearly in the 300 to 1500 µM range (Figure 6) following the linear regression equation as shown. The developed sensor didn't show a clear response for 3-MCPD concentration in the range 50 to 250 µM (calibration curve not shown). Hence the linear range was observed from 300 to 1500 µM. The sensitivity of the developed sensor, obtained from the gradient of the calibration curve was found to be 0.011 mV/µM.

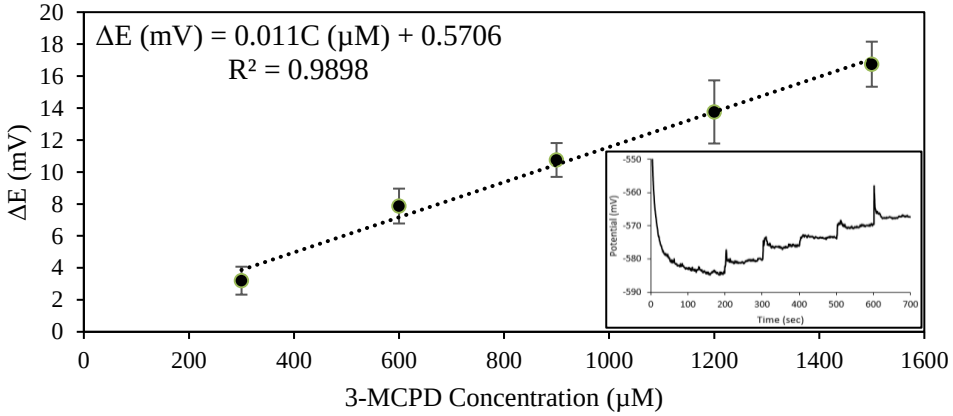


Figure 6: Calibration curve for 3-MCPD detection on MWCNT/AuNP/MIP/SPCE under optimal conditions (Error bars indicate S.D). (Inset: Potentiometric response of 3-MCPD in the linear range)

The detection limit (LOD) and the quantification limit (LOQ) of the developed sensor were evaluated according to the response standard deviation and slope based method described by International Conference on Harmonization (ICH) using following equations (Guideline, 2005; Shrivastava & Gupta, 2011).

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} \quad (2)$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve. The estimate of σ was obtained by the standard deviation of the y-intercept of the regression line i.e. standard error of the estimate. Accordingly, the LOD of the sensor was found to be 192.53 µM and the LOQ was 583.42 µM.

1,3-DCP, 1,2-propanediol, and glycerol which are close analogues of 3-MCPD (Sun *et al.*, 2014) and D-glucose which is a common saccharide having a high affinity towards boronic acid derivatives were selected as interferents to evaluate their influence on 3-MCPD detection by the sensor. For comparison of selectivity, interferent tests were done on both imprinted and non-imprinted electrodes. The competitive binding ability of the sensor with 3-MCPD in the presence of interfering substances was tested by adding 300 µM of 3-MCPD together with 15 mM (50-fold excess) of one interferent. Similarly, all four interferents were tested separately. As presented in Figure 7 (3-MCPD is included for comparison), a clear distinction was observed between MIP and NIP response. The MIP electrodes showed a positive potential change at different levels for each interferent while the NIP electrodes showed a negative response to all the interferents. The change in polarity between MIP and NIP in the presence of interferents clearly differentiate the ability of the MIP electrode to selectively detect 3-MCPD in the presence of challenging interferent molecules.

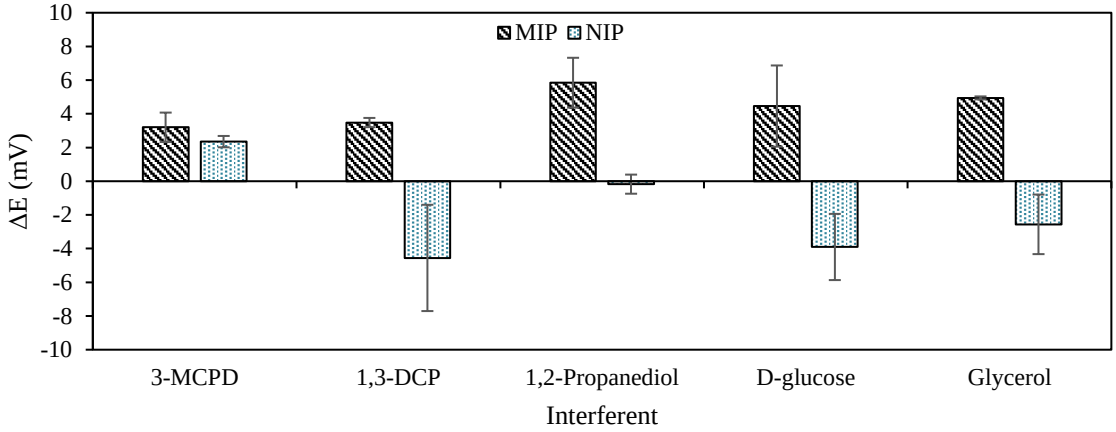


Figure 7: Potentiometric response upon the addition of 300 µM 3-MCPD + 15 mM interferent (Error bars indicate S.D)

The reproducibility of the sensor was determined by measuring the potential change (ΔE) upon the sequential addition of 3-MCPD in the range 300-1500 µM for 3 fresh electrodes made using the same procedure (Table 1). Except for 300 µM, the relative standard deviation was less than 15% in the range 600 – 1500 µM. This indicates that the electrode modification and 3-MCPD detection is moderately reproducible.

Table 2: Potential change determined by 3 fresh electrodes

3-MCPD Concentration (µM)	ΔE (mV)	RSD (%)
300	3.20 ± 0.87	27.31
600	7.87 ± 1.09	13.86
900	10.75 ± 1.06	9.84
1200	13.76 ± 1.97	14.35
1500	16.74 ± 1.41	8.42

The stability of the sensor was investigated over a storage period of 20 days by storing the sensor in a transparent air tight zip lock bags at room temperature. The potential change (ΔE) upon the sequential addition of 3-MCPD in the range 300 – 1500 µM was measured on the 10^{th} and 20^{th} day of storage and the response was compared with the freshly modified sensor. Statistical analysis indicated that there was no significant change (p >.05) in response at all concentration levels even after 20 days of storage. This testifies to the good stability of the developed sensor under normal room temperature conditions.

4 CONCLUSION

A commercially available low-cot screen printed carbon electrode (SPCE) was modified with MWCNT, AuNPs and molecularly imprinted poly(3-aminophenylboronic acid) for selective detection of 3-monochloropropane-1,2-diol (3-MCPD). The presented method created 3-MCPD imprinted 3-APBA polymer film on the SPCE which was specific to 3-MCPD in the presence of selected competitive interferents. Fabrication and measurement conditions were optimized for the concentration of MWCNT, AuNP electrodeposition cycles, a number of electropolymerization cycles and the response behaviour were assessed for the PBS pH range of 6.2 to 8.0. A calibration curve was developed for the linear range of the sensor from 300 to 1500 µM and the sensitivity was determined as 0.011 mV/µM. The LOD and LOQ of the developed sensing platform were found to be 192.53 µM and 583.42 µM respectively. The sensor demonstrated moderate reproducibility and high stability under normal room temperature conditions. The sensing platform developed in this study could be further modified for more sensitive mediator free potentiometric sensing of 3-MCPD in real food sample applications.

5 ACKNOWLEDGEMENTS

AIT Fellowship offered to Niroshan Chinthaka Gunawardena by the Asian Institute of Technology, Thailand is thankfully acknowledged.

REFERENCES

- Badhulika, S., & Mulchandani, A. (2015). Molecular imprinted polymer functionalized carbon nanotube sensors for detection of saccharides. *Applied Physics Letters*, *107*(9), 093107.
- Codex Alimentarius Commission. (2001). Joint FAO/WHO food standards programme. Codex Committee on Food Additives and Contaminants. Thirty-third Session. Position Paper on Chloroproponals.
- Commission, E. C. (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. 2006R1881-EN-01.09. 2014-014.001-1.
- Dago, À., Navarro, J., Ariño, C., Díaz-Cruz, J. M., & Esteban, M. (2015). Carbon nanotubes and graphene modified screen-printed carbon electrodes as sensitive sensors for the determination of phytochelatins in plants using liquid chromatography with amperometric detection. *Journal of Chromatography A*, 1409, 210-217.
- Deore, B., & Freund, M. S. (2003). Saccharide imprinting of poly (aniline boronic acid) in the presence of fluoride. *Analyst*, *128*(6), 803-806.
- Devkota, L., Nguyen, L. T., Vu, T. T., & Piro, B. (2018). Electrochemical determination of tetracycline using AuNP-coated molecularly imprinted overoxidized polypyrrole sensing interface. *Electrochimica Acta*.
- Do, P. T., Do, P. Q., Nguyen, H. B., Nguyen, V. C., Tran, D. L., Le, T. H., *et al.* (2014). A highly sensitive electrode modified with graphene, gold nanoparticles, and molecularly imprinted over-oxidized polypyrrole for electrochemical determination of dopamine. *Journal of Molecular Liquids*, *198*, 307-312.
- Guideline, I. H. T. (2005, November). Validation of analytical procedures: text and methodology Q2 (R1). In International Conference on Harmonization, Geneva, Switzerland (pp. 11-12).
- Hezard, T., Fajerwerg, K., Evrard, D., Collière, V., Behra, P., & Gros, P. (2012). Gold nanoparticles electrodeposited on glassy carbon using cyclic voltammetry: Application to Hg (II) trace analysis. *Journal of Electroanalytical Chemistry*, 664, 46-52.
- IARC. (2017). Agents classified by the IARC monographs, Volumes 1–109.
- Lamas-Ardisana, P. J., Queipo, P., Fanjul-Bolado, P., & Costa-García, A. (2008). Multiwalled carbon nanotube modified screen-printed electrodes for the detection of p-aminophenol: Optimisation and application in alkaline phosphatase-based assays. *Analytica Chimica Acta*, 615(1), 30-38.
- Lee, B. Q., & Khor, S. M. (2015). 3-Chloropropane-1, 2-diol (3-MCPD) in Soy Sauce: A Review on the Formation, Reduction, and Detection of This Potential Carcinogen. *Comprehensive Reviews in Food Science and Food Safety*, 14(1), 48-66.
- Li, J., Liu, L., Wang, P., & Zheng, J. (2014). Potentiometric detection of saccharides based on highly ordered poly (aniline boronic acid) nanotubes. *Electrochimica Acta*, *121*, 369-375.
- Muhammad, A., Hajian, R., Yusof, N. A., Shams, N., Abdullah, J., Woi, P. M., & Garmestani, H. (2018). A screen printed carbon electrode modified with carbon nanotubes and gold nanoparticles as a sensitive electrochemical sensor for determination of thiamphenicol residue in milk. *RSC Advances*, 8(5), 2714-2722.
- OEHHA. (2010). Evidence on the Carcinogenicity of 3-Monoghloropropane-1,2-diol (3-MCPD; a-Chlorohydrin). California Environmental Protection Agency.
- Rezaei, B., Boroujeni, M. K., & Ensafi, A. A. (2014). A novel electrochemical nanocomposite imprinted sensor for the determination of lorazepam based on modified polypyrrole@ sol-gel@ gold nanoparticles/pencil graphite electrode. *Electrochimica Acta*, 123, 332-339.

- Shoji, E., & Freund, M. S. (2002). Potentiometric saccharide detection based on the pKa changes of poly (aniline boronic acid). *Journal of the American Chemical Society*, *124*(42), 12486-12493.
- Shoji, E., & Freund, M. S. (2001). Potentiometric sensors based on the inductive effect on the pKa of poly (aniline): a nonenzymatic glucose sensor. *Journal of the American Chemical Society*, *123*(14), 3383-3384.
- Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists*, 2(1), 21-21.
- Sienkiewicz, P. A., & Roberts, D. C. (1980). Chemical affinity systems—I: pH dependence of boronic acid-diol affinity in aqueous solution. *Journal of Inorganic and Nuclear Chemistry*, 42(11), 1559-1575.
- Springsteen, G., & Wang, B. (2002). A detailed examination of boronic acid–diol complexation. Tetrahedron, 58(26), 5291-5300.
- Sun, X., Zhang, L., Zhang, H., Qian, H., Zhang, Y., Tang, L., & Li, Z. (2014). Development and application of 3-chloro-1, 2-propandiol electrochemical sensor based on a polyaminothiophenol modified molecularly imprinted film. *Journal of agricultural and food chemistry*, 62(20), 4552-4557.
- Van Duin, M., Peters, J. A., Kieboom, A. P. G., & Van Bekkum, H. (1984). Studies on borate esters 1: the pH dependence of the stability of esters of boric acid and borate in aqueous medium as studied by 11B NMR. *Tetrahedron*, 40(15), 2901-2911.
- Velíšek, J., Davidek, J., Hajšlová, J., Kubelka, V., Janícek, G., & Mánková, B. (1978). Chlorohydrins in protein hydrolysates. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 167(4), 241-244.
- Viet, N. X., & Takamura, Y. (2016). Electrodeposited Gold Nanoparticles Modified Screen Printed Carbon Electrode for Enzyme-Free Glucose Sensor Application. *VNU Journal of Science: Natural Sciences and Technology*, 32(4).
- Wang, C., Wang, Q., Zhong, M., & Kan, X. (2016). Boronic acid based imprinted electrochemical sensor for rutin recognition and detection. *Analyst*, *141*(20), 5792-5798.
- Wu, S., Guo, H., Wang, L., Xin, Y., Cheng, Y., & Fan, W. (2017). An ultrasensitive electrochemical biosensing platform for fructose and xylitol based on boronic acid-diol recognition. *Sensors and Actuators B: Chemical*, *245*, 11-17.
- Yan, J., Springsteen, G., Deeter, S., & Wang, B. (2004). The relationship among pKa, pH, and binding constants in the interactions between boronic acids and diols—it is not as simple as it appears. *Tetrahedron*, 60(49), 11205-11209.
- Zeng, Y., Zhu, Z., Du, D., & Lin, Y. (2016). Nanomaterial-based electrochemical biosensors for food safety. *Journal of Electroanalytical Chemistry*, 781, 147-154.
- Zhong, M., Teng, Y., Pang, S., Yan, L., & Kan, X. (2015). Pyrrole–phenylboronic acid: A novel monomer for dopamine recognition and detection based on imprinted electrochemical sensor. *Biosensors and Bioelectronics*, *64*, 212-218.
- Zhou, Y., Dong, H., Liu, L., Liu, J., & Xu, M. (2014). A novel potentiometric sensor based on a poly (anilineboronic acid)/graphene modified electrode for probing sialic acid through boronic acid-diol recognition. *Biosensors and Bioelectronics*, *60*, 231-236.

A NMR-Based Metabolomics Approach for Reducing Food Losses: The Example of Minced Pork Meat

Emilie Cauchie[1]*, Nicolas Korsak[1], Justine Leenders[2]*, Ghislain Baré[1], Assia Tahiri[1], Laurent Delhalle[1], Pascal de Tullio[2] and Daube Georges[1]

[1] University of Liège, Fundamental and Applied Research for Animal & Health (FARAH), Faculty of Veterinary Medicine, Food Science Department, Quartier Vallée, Avenue de Cureghem 10, B-4000 Liège, Belgium.

[2] University of Liège, Center for Interdisciplinary Research on Medicines (CIRM), Metabolomics group, Quartier Hôpital, Avenue Hippocrate 15, B-4000 Liège, Belgium

* Corresponding Authors: ecauchie@uliege.be and jleenders@uliege.be

ABSTRACT

In Europe, the losses of initial meat production represent 20% and more than half of this occurs at animal production, slaughtering, processing and distribution step. In order to control food waste, studies have highlighted the importance of monitoring the microbial diversity of food products because spoilage by bacteria that contaminate the food matrix is a major issue. As such, the combination of metabolomics data with other complementary approaches (classical microbiology and quality parameters) can gives the opportunity to gain deeper insights into and have a better comprehension of the spoilage mechanisms. The aim of the current study was to assess meat spoilage through the evolution of bacterial counts and changes in the metabolic profile of minced pork meat using Nuclear Magnetic Resonance (NMR) based metabolomics. Microbiological assessment, pH measurements, gas composition and metabolomics analysis were carried out in meat samples stored under food wrap and under modified atmosphere packaging (70% O_2 – 30% CO_2) at 4, 8 and 12 °C during 13 days. All samples were irradiated and then inoculated separately with three dominant bacterial species: *Brochothrix thermosphacta*, *Leuconostoc gelidum* and *Pseudomonas fragi*. For all conditions, non-inoculated samples were also stored. Analysis were carried out at day 0 and at day 13 for metabolomics analysis, and each day for all others measurements. The multivariate analysis (PLS-DA) reveals a clear discrimination between: (i) the non-inoculated product at day 0 and at day 13, (ii) the inoculated and non-inoculated samples, (iii) the type of bacterium, and (iv) the packaging conditions. It can be observed that the type of bacterium inoculated had a higher impact on the metabolome than that the packaging conditions. Moreover, some metabolites are significantly increased: acetate and glycerol for *B. thermosphacta*, betaine and lactate for *L. gelidum*, threonine and glycine for *P. fragi*. Exploration of the correlations of NMR-based metabolomics results with others microbial parameters suggested their use as possible spoilage tool to provide information on minced pork meat spoilage and to follow intrinsically the evolution of the metabolomics pattern linked to a specific bacterium in a complex bacterial ecosystem.

KEYWORDS: Meat spoilage, NMR Metabolomics, *Brochothrix thermosphacta*, *Pseudomonas fragi*, *Leuconostoc gelidum*.

1 INTRODUCTION

Among the reasons for food loss and waste, spoilage by bacteria that contaminate the food matrix and are able to develop during transformation steps and storage is a major issue (Lipsinki *et al.*, 2013; Remenant *et al.*, 2015). It is well established that spoilage of meat is the result of decomposition and formation of metabolites caused by the growth and enzymatic activity of microorganisms (Argyri *et al.*, 2015), highlighting the importance of managing the quality of food products. New "omics" approaches and more specifically metabolomics, which deals with the study of the metabolites profiles of samples, is a relatively new investigate tool in food science. Recently, application of NMR spectroscopy coupled

with multivariate analysis has been used to obtain metabolite profiling of various kinds of food including salmon, meat, honey, milk, olive oil, wine, tea and others plants (Jung *et al*, 2010).

NMR spectroscopy and more specifically proton NMR is, together with mass spectrometry, the most widely used analytical platform for metabolomics analysis. It has proved to be an attractive technique offering in a single experiment an overview of a wide range of compounds present in a matrix by detecting all ^{1}H containing metabolites with concentrations above tens of micromolar level (Chen *et al.*, 2016; Yuan *et al.*, 2017). NMR-based metabolomics can be potentially useful for identifying variation between the metabolic profiles linked to typical processing and storage conditions, and can be used to identify specific fingerprinting that could be correlated with the samples status (Boffo *et al.*, 2012; Charlton *et al.*, 2002). However, among all these studies only a few have been performed on foods (Piras *et al.*, 2013), and particularly on meat (Castejon *et al.*, 2015; Ritota *et al.*, 2012). Although water content of meat and abundance of nutrients available on the surface make it one of the most perishable foods (Ercolini *et al.*, 2011), no metabolite profiles from inoculated meat by putatively spoilage microorganisms have been reported to date (Zanardi *et al.*, 2015). Then, it is expected that the combination of metabolomics data with other complementary approaches (classical microbiology and quality parameters) can gives the opportunity to gain deeper insights into, and have a better comprehension of the spoilage mechanisms (Consonni and Cagliani, 2008; Mannina *et al.*, 2012).

According to this, this work aims at studying by a NMR-based metabolomics approach the changes in metabolites profiles of minced pork meat during shelf life product and meat spoilage. For this purpose, non-inoculated and inoculated samples, respectively with *Brochothrix thermosphacta*, *Pseudomonas fragi* and *Leuconostoc gelidum* were collected, considered as one of commonly specific spoilage organism (SSO) (Miks-Krajnik *et al.*, 2016). Metabolites profiling were then measured and analysed according to modifications of some environmental parameters of storage (temperature and food packaging). These parameters are important factors that can influence the biochemical components of samples. The metabolomics data were compared with others classical methods used to follow the bacterial evolution in complex ecosystem (classical microbiology, pH values and gas composition measurements).

2 MATERIAL AND METHODS

2.1 Food Samples and Selection of Bacterial Isolates

The isolates used in this study were previously isolated from Belgian minced pork meat (three batches from four producers) at the end of their use-by date by 16S rRNA metagenetics and bacterial counts by classical microbiology. Three of the natural predominant bacteria isolated at the end of the shelf life, represented more than 50% of the natural microbiota, were identified by sequencing of their 16S rRNA genes and used for the challenge-tests: *Brochothrix thermosphacta* (MM008), *Leuconostoc gelidum* (MM045) and *Pseudomonas fragi* (*Pseudomonas fragi* MM014 and MM015). Bacterial isolates were stored at −80°C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, isolates were transferred from the −80 °C culture collection to Brain Heart Infusion (BHI) broth for 48 h at 22 °C. The cultures were incubated overnight at 4°C before inoculation.

Fresh minced pork meat packed under air with a food wrap film were obtained from a local Belgian manufacturer. The water activity of this product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.02. Food samples were irradiated by gamma irradiation at 10 kGy (Sterigenics, Fleurus, Belgium) and were stored until used at -20 °C.

2.2 Challenge-Tests

The products were inoculated, in triplicate, by adding sterile water containing each individually of the three bacterial isolates with the goal of reaching an approximatively global concentration of 3.00 log colony forming units (log CFU/g on the product) (n=465). The samples were mixed by a Kenwood mixer (Kenwood Belgium, Mechelen, Belgium) for 2 min in speed 2. Non-inoculated control samples were homogenized by adding the same quantity of sterile water only, in triplicate (n=36). Minced pork meat was then packed (50 g) in a tray (PP/EVOH/PP) under modified atmosphere (MAP, CO_2 30% / O_2 70%) (Olympia V/G, Technovac, Italy) and under food wrap packing (FW) (cling film). For this

study, a short 13 days shelf life was evaluated for the minced pork meat. Inoculated samples were stored at different constant temperatures of 4 °C (± 1 °C), 8 °C (± 1 °C) and 12 °C (± 1 °C) in temperature-controlled incubators. Control samples were only stored at the first day of inoculation (day 0) and at day 13.

2.3 Conventional Microbiological Method

Each day during the 13-days storage period, 25 g of product were put into a Stomacher bag with a mesh screen liner (80 µm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Physiological water (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in peptone water (1 g/L peptone, 8.5 g/L sodium chloride) were prepared for microbiological analysis and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis in duplicate (Spiral plater, DW Scientific, England). A total count was made on Plate Count Agar (PCA) at 22 °C for 48 h for the psychrotrophic aerobic plate count (PAPC), using the modified method specified by the International Organization for Standardization [ISO (2013, ISO 4833-2)].

2.4 Samples pH Measurements and Gas Composition Values

The pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne) at day 0 and day 13. For all samples stored in modified atmosphere packaging measurements of the composition in gas was monitored daily until day 13 (CheckMate 3, Dansensor, France). Using Excel, t-student test was used to evaluate statistical differences between samples measurements, all tests were considered as significant for a p-value of < 0.05.

2.4 Samples Preparation for NMR Analysis

^{1}H-NMR analysis of minced pork meat samples was realized at day 0 and day 13, in five repetitions (n=240), for inoculated and non-inoculated samples. 100 mg of each meat samples were reconstituted in an eppendorf tube by adding 500 µl D_2O (DPB, pH 7.4). The mixture was homogenized with a vortex during 1 min and then centrifugated 2x30 sec at 5000 rpm. 300 µl of D_2O were added and the mixture was homogenizing with a vortex 1 min and then leave for 15 min. A last centrifugation step during 15 min at 13000 rpm was performed and the mixture was transferred to a NMR tube for the analysis of the supernatant (650 µl) by ^{1}H-NMR.

2.4 NMR Measurements

All samples were recorded at 298 K on a Bruker Avance spectrometer operating at 500 MHz for the proton signal acquisition. The instrument was equipped with a 5 mm TCI cryoprobe with a Z-gradient. Maleic acid was used as internal standard for quantification and trimethylsilyl-3-propionic acid-*d*4 (TMSP) for the zero calibration. 650 µl of meat supernatant (prepared as described before) were supplemented with 100 µl of deuterated phosphate buffer, 100 µl of a 5 mM solution of maleic acid and 5 µl of a 10 mg/ml TMSP solution. ^{1}H-NMR spectra were acquired using a CPMG relaxation-editing sequence with presaturation for meat samples. The CPMG experiment used a RD-90-(t-180-t)n-sequence with a relaxation delay (RD) of 2 s, a spin echo delay (t) of 400 ms and the number of loops (n) equal to 80. The water suppression pulse was placed during the relaxation delay (RD). The number of transients was typically 32 and a number of 4 dummy scans was chosen. The data were processed with the Bruker Topspin 3.2 software with a standard parameter set. Phase and baseline corrections were performed manually over the entire range of the spectra and the δ scale was calibrated to 0 ppm using the internal standard TMSP.

2.4 Multivariate Analysis

For statistical analysis, optimized ^{1}H-NMR spectra were automatically baseline-corrected and reduced to ASCII files using AMIX software (version 3.9.14; Bruker). The spectral intensities were normalized to total intensities and reduced to integrated regions of equal width (0.04 ppm) corresponding to the 0.5–10.00 ppm region. Because of the residual signals of water and maleic acid, regions between 4.7 and 5 ppm (water signal) and 5.6–6.2 ppm (maleic acid signal) were removed before analysis. The reduced and normalized NMR spectral data were imported into SIMCA (version 13.0.3, Umetrics AB, Umea Sweden). Pareto scaling was applied to bucket tables and discriminant analysis (DA) such as PCA (Principal Component Analysis), PLS-DA (Partial Least Squares Discriminant Analysis), OPLS-DA (orthogonal partial least squares discriminant analysis) and PLS (Partial Least Square) regression were performed. SIMCA was used to generate all PCA, PLS, PLS-DA, and OPLS-DA models and plots. PCA was only used to detect possible outliers and determine intrinsic clusters within the data set, while PLS-DA maximized the separation and OPLS-DA facilitated the graphic visualization of differences and similarities between groups. The quality of OPLS-DA models was determined by the goodness of fit (R^2) and the predictability was calculated on the basis of the fraction correctly predicted in one-seventh cross-validation (Q^2).

2.4 Metabolite Identification

From PLS-DA loading plots, metabolites with higher loadings were identified. Signals with values of Variable Importance in Projection (VIP) higher than 1 were considered as significant, and further validated using t-test with Metaboanalyst (http://www.metaboanalyst.ca). Metabolite identification was next performed using the open-access database NMR suite 8.1 (Chenomx inc., Edmonton, Canada), the free web-based tool HMDB (http://www.hmdb.ca) and tables. Each metabolite identified was finally confirmed by performing peak correlation plots from 2D-NMR spectra (COSY and HSQC)

3 RESULTS

3.1 Microbial Counts

Tables 1 and 2 show the PCA results from challenge tests at different temperature (4, 8 and 12 °C) and for the two packaging (MAP and FW). As expected, the storage temperature and the packaging conditions have a strong impact on the bacterial evolutions. At 8 °C, *Pseudomonas fragi* reach approximatively 10.00 and 8.30 log CFU/g in FW and MAP, respectively. A high growth rate and a more rapidly reached stationary phase are also correlated to the FW packaging and the highest storage temperatures. No bacterial growth is observed on PCA for the control samples (limit detection < 3.00 log CFU/g, results not shown).

Table 1: Microbiological counts for challenge tests in food wrap (FW) packaging, at different temperature, for a 13-days shelf life.

Days	Bacterial species		
	Brochothrix thermosphacta	*Pseudomonas fragi*	*Leuconostoc gelidum*
	4°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	3.08 ± 0.10	3.43 ± 0.11	4.07 ± 0.01
2	3.76 ± 0.07	4.52 ± 0.23	4.38 ± 0.01
3	4.54 ± 0.12	5.64 ± 0.19	4.61 ± 0.12
4	- [a]	- [a]	- [a]
5	- [a]	- [a]	- [a]
6	7.24 ± 0.11	- [a]	6.17 ± 0.05
7	7.74 ± 0.17	9.45 ± 0.13	- [a]
8	7.63 ± 0.10	9.51 ± 0.07	- [a]
9	8.17 ± 0.33	- [a]	- [a]
10	7.68 ± 0.15	9.90 ± 0.29	8.62 ± 0.09
11	- [a]	- [a]	- [a]
12	- [a]	- [a]	- [a]
13	7.90 ± 0.15	10.21 ± 0.03	8.42 ± 0.06
	8°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	6.76 ± 0.04	3.86 ± 0.17	4.58 ± 0.08
2	7.49 ± 0.11	5.36 ± 0.03	5.84 ± 0.02
3	8.25 ± 0.07	7.69 ± 0.17	- [a]
4	8.51 ± 0.10	9.04 ± 0.05	7.57 ± 0.10
5	8.58 ± 0.06	9.67 ± 0.03	- [a]
6	8.85 ± 0.02	- [a]	8.61 ± 0.13
7	8.77 ± 0.15	9.62 ± 0.15	- [a]
8	9.05 ± 0.03	10.34 ± 0.24	8.73 ± 0.07
9	8.79 ± 0.21	10.39 ± 0.40	- [a]
10	- [a]	10.11 ± 0.28	8.84 ± 0.09
11	- [a]	- [a]	- [a]
12	- [a]	- [a]	- [a]
13	9.00 ± 0.01	10.15 ± 0.17	8.77 ± 0.30
	12°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	7.68 ± 0.08	4.93 ± 0.15	5.38 ± 0.01
2	8.29 ± 0.13	- [a]	6.84 ± 0.13
3	8.66 ± 0.04	9.81 ± 0.04	8.35 ± 0.09
4	8.99 ± 0.09	9.85 ± 0.29	7.56 ± 0.01
5	9.01 ± 0.23	9.95 ± 0.34	- [a]
6	9.11 ± 0.10	10.15 ± 0.82	8.64 ± 0.13
7	8.81 ± 0.28	10.26 ± 0.08	- [a]
8	9.03 ± 0.03	10.14 ± 0.10	- [a]
9	8.91 ± 0.16	- [a]	- [a]
10	- [a]	9.87 ± 0.19	8.82 ± 0.23
11	- [a]	- [a]	- [a]
12	- [a]	- [a]	- [a]
13	9.27 ± 0.08	9.80 ± 0.42	8.62 ± 0.18

[a] no analysis performed for the day.

Table 2: Microbiological counts for challenge tests in modified atmosphere (MAP) packaging, at different temperature, for a 13-days shelf life.

Days	Bacterial species		
	Brochothrix thermosphacta	*Pseudomonas fragi*	*Leuconostoc gelidum*
	4°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	- [a]	- [a]	4.18 ± 0.09
2	- [a]	3.48 ± 0.06	- [a]
3	2.17 ± 0.30	- [a]	- [a]
4	- [a]	- [a]	6.31 ± 0.17
5	- [a]	3.90 ± 0.11	- [a]
6	4.11 ± 0.01	4.87 ± 0.34	6.84 ± 0.06
7	4.01 ± 0.14	4.55 ± 0.12	7.85 ± 0.01
8	4.35 ± 0.03	- [a]	- [a]
9	5.24 ± 0.05	- [a]	7.78 ± 0.21
10	4.99 ± 0.12	- [a]	- [a]
11	- [a]	- [a]	- [a]
12	- [a]	4.73 ± 0.01	8.00 ± 0.10
13	5.43 ± 0.06	4.90 ± 0.01	8.39 ± 0.12
	8°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	- [a]	3.52 ± 0.01	4.75 ± 0.03
2	5.88 ± 0.10	4.16 ± 0.05	- [a]
3	6.11 ± 0.11	- [a]	- [a]
4	7.11 ± 0.02	- [a]	8.06 ± 0.01
5	7.86 ± 0.10	5.41 ± 0.08	- [a]
6	8.21 ± 0.04	6.33± 0.07	8.38 ± 0.05
7	8.43 ± 0.11	6.52 ± 0.14	8.49 ± 0.16
8	8.43 ± 0.16	- [a]	- [a]
9	8.41 ± 0.10	6.59 ± 0.17	8.85 ± 0.01
10	8.38± 0.16	- [a]	- [a]
11	- [a]	- [a]	- [a]
12	7.86 ± 0.07	7.83 ± 0.13	- [a]
13	8.76 ± 0.03	8.37 ± 0.08	8.75 ± 0.19
	12°C		
0	3.84 ± 0.03	3.15 ± 0.59	4.00 ± 0.02
1	- [a]	4.47 ± 0.07	8.32 ± 0.15
2	7.10 ± 0.04	6.08 ± 0.03	7.28 ± 0.01
3	7.76 ± 0.23	- [a]	- [a]
4	8.35 ± 0.04	- [a]	8.35 ± 0.06
5	8.58 ± 0.06	- [a]	- [a]
6	8.40 ± 0.12	9.42 ± 0.28	8.36 ± 0.09
7	8.44 ± 0.07	9.58 ± 0.23	8.64 ± 0.10
8	8.32 ± 0.03	- [a]	- [a]
9	9.16 ± 0.08	9.80 ± 0.41	8.89 ± 0.07
10	8.67 ± 0.40	- [a]	- [a]
11	- [a]	- [a]	- [a]
12	8.83 ± 0.02	9.87 ± 0.06	- [a]
13	8.71 ± 0.06	9.85 ± 0.14	8.87 ± 0.11

[a] no analysis performed for the day.

3.2 pH and Gas Measurements

Figure 1 shows the comparison between pH measurements for control and inoculated samples at day 13. A significant increase of pH is only observed for *Pseudomonas fragi*, which reach a higher pH value at day 13 (7.54 ± 0.76, n=5) compared to control samples (5.79 ± 0.05, n=10). There were no differences of pH values between FW and MAP conditions for control and inoculated samples.

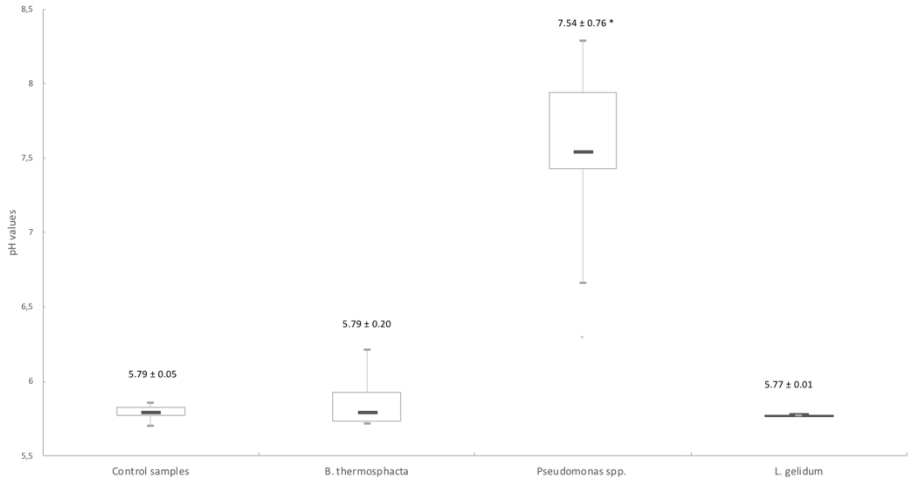


Figure 1: Comparison of pH values for control samples (non-inoculated sterile products) and inoculated products at day 13 for all packaging conditions, * significant statistical difference ($p < 0.05$).

It was also observed a relatively stable composition of MAP packaging in control samples at day 0 (29.6 ± 0.53, n=13) and at day 13 (30.1 ± 1.62, n=12). But it can be observed significant statistical differences for each challenge test, at day 13, according to the temperature condition (Figure 2). The highest values were obtained with *Pseudomonas fragi*, which reached a CO_2 values of 100.0 ± 0.1% at 12°C.

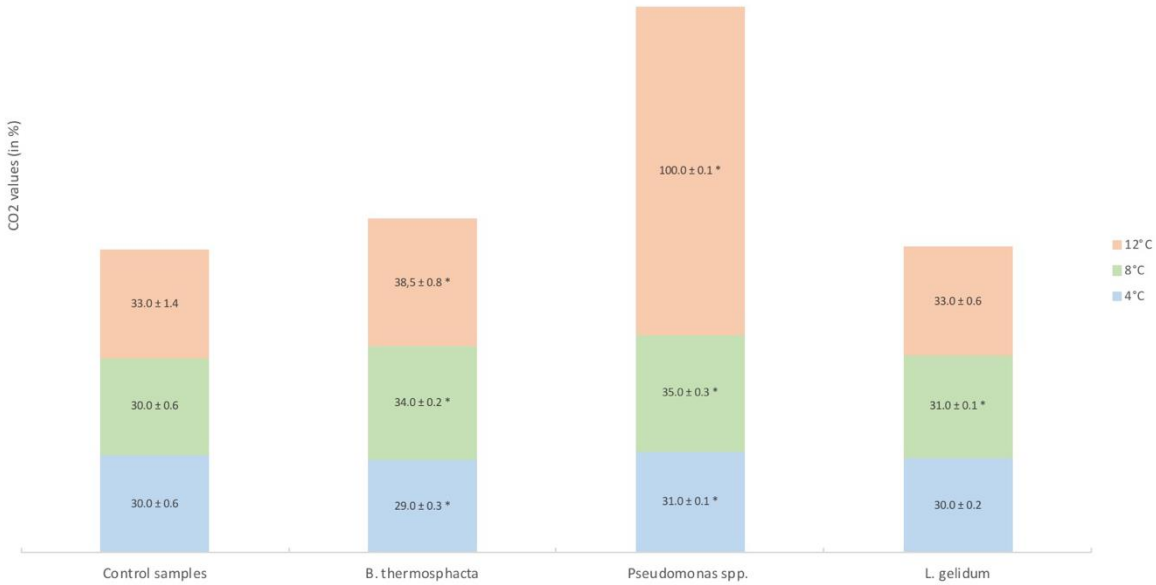


Figure 2: Comparison of CO_2 measurements for control samples (non-inoculated sterile products) and inoculated products at day 13 for modified atmosphere packaging conditions, * significant statistical difference ($p < 0.05$).

3.3 NMR Patterns and Metabolites

The multivariate analysis (PLS-DA) reveals a clear discrimination between: (i) the non-inoculated products at day 0 and 13 (Figure 3), (ii) the inoculated and non-inoculated samples (Figure 4), (iii) the type of bacterium inoculated on the samples (Figure 5), and (iv) the packaging conditions (Figure 6).

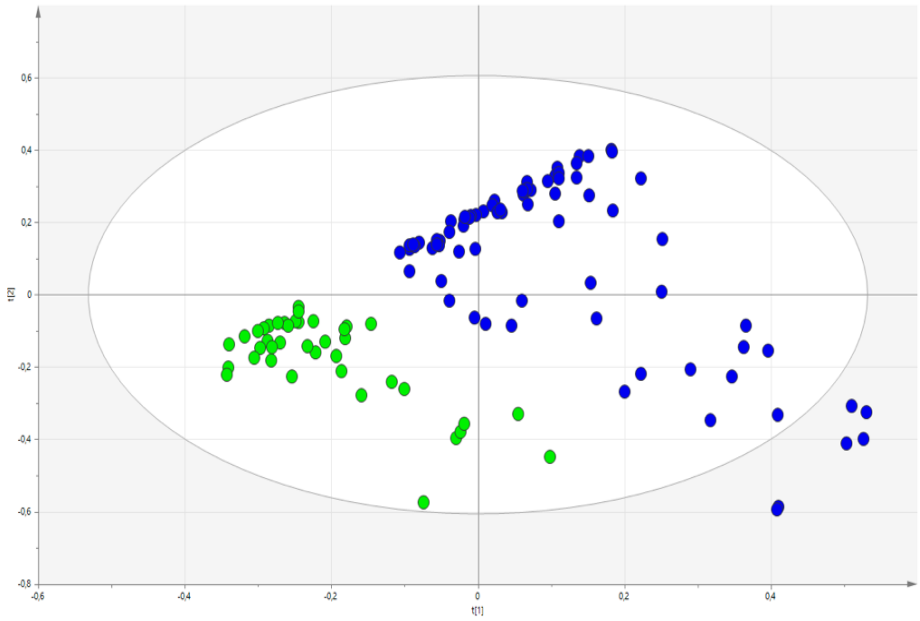


Figure 3: Comparison of metabolomic profiles between non-inoculated samples at day 0 (green circles) and day 13 (blue circles) (R^2=0.925, Q^2=0.903).

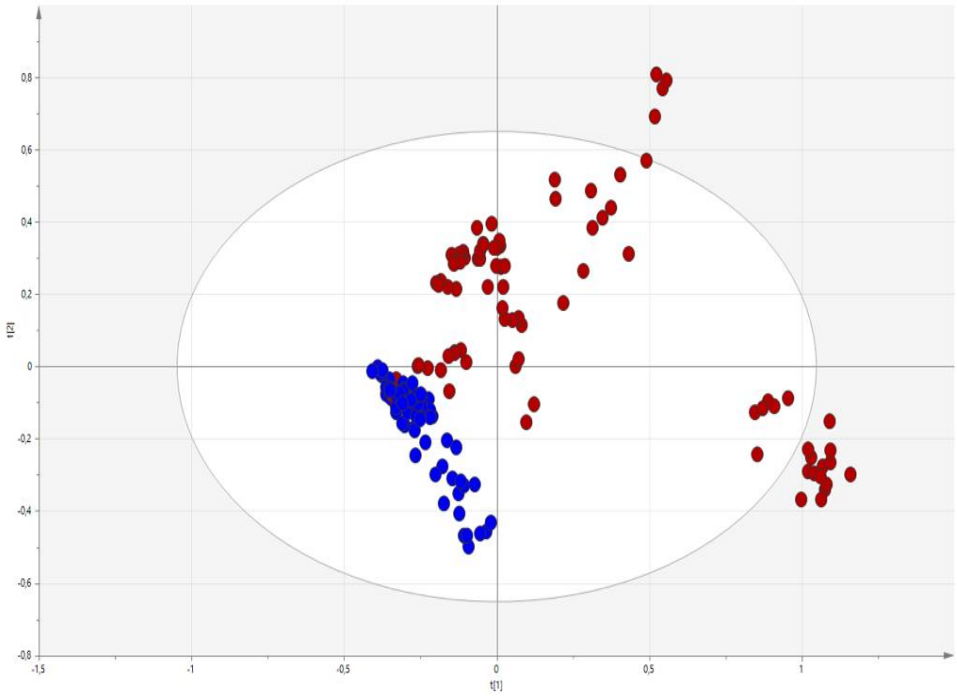


Figure 4: Comparison of metabolomic profiles between non-inoculated (blue circles) and inoculated samples (red circles) at day 13 (R^2=0.858, Q^2=0.838).

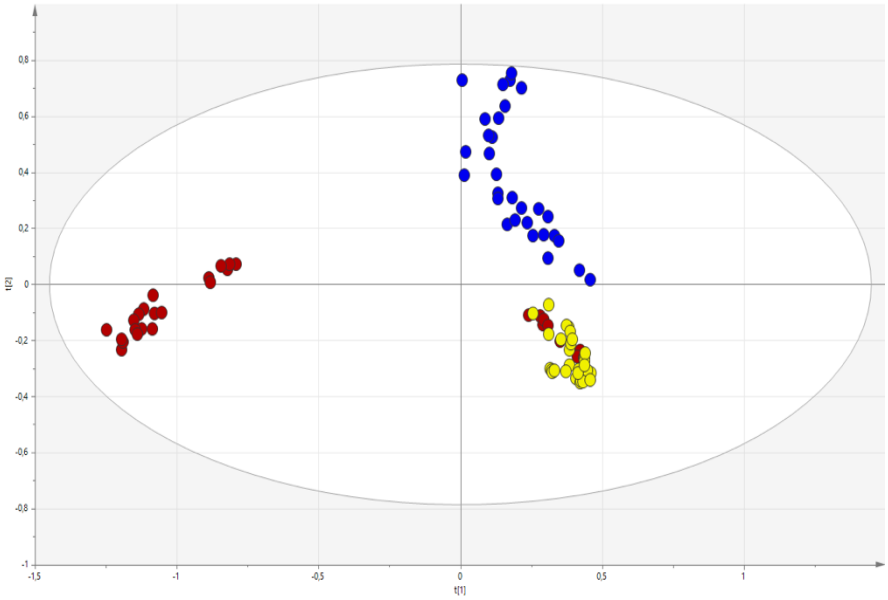


Figure 5: Comparison of metabolomic profiles at day 13 between each inoculated bacterium on samples: *Brochothrix thermosphacta* (blue circles), *Pseudomonas fragi* (red circles) and *Leuconostoc gelidum* (yellow circles) (R^2=0.842, Q^2=0.830).

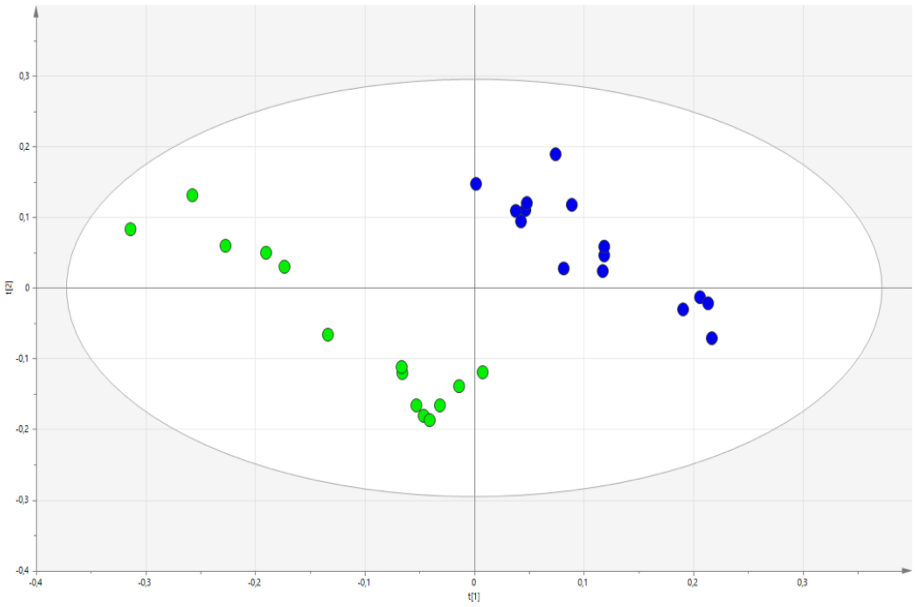


Figure 6: Comparison of metabolomic profiles between each packaging at day 13: modified atmosphere (bleu circles) and food wrap (green circles) (R^2=0.985, Q^2=0.967).

It can be observed that the type of bacterium inoculated had a higher impact on the metabolome than that of the packaging conditions.

Moreover, some metabolites are significantly increased: acetate and acetoin for *B. thermosphacta*, lipoproteins and glutamine for *Pseudomonas fragi*, betaine and lactate for *L. gelidum*. But this part of study needs to be studied more deeply.

4 DISCUSSION

Exploration of the correlations of NMR-based metabolomics results with others microbial parameters suggested their use to provide information on minced pork meat spoilage. Spoilage occurs when the formation of off-flavors, off-odors, discoloration, slime, or any other changes in physical

appearance or chemical characteristics make the food unacceptable levels (Ercolini *et al.*, 2011). According to this, the qualitative and quantitative analyses of metabolic compounds present as a consequence of microbial activity have been considered as a more integrated holistic approach, in which meat quality can be estimated regardless of storage conditions (e.g. temperature, type of packaging) (Argyri *et al.*, 2015). These informations can also be added to other microbial results such as microbiological counts, pH changes and gas composition.

In the food wrap packaging, *Pseudomonas fragi* reached the highest microbiological counts at the end of day 13. And all bacteria reach stationary phase in modified atmosphere packaging. A high growth rate and a more rapidly reached stationary phase are also correlated with the highest storage temperatures. These results are of interest because these bacteria are responsible for unpleasant odours and flavours making the product inedible and are considered as specific spoilage organisms in meat products (Pothakos *et al.*, 2015; Saraoui *et al.*, 2017). *Pseudomonas fragi* also shows the highest values of pH comparatively to the control samples, and the modifications of CO_2 in the MAP packaging is also more important at 12°C for *P. fragi*. The change in pH of products is usually a good index for quality assessment. Indeed, it is commonly related to the accumulation of lactic acid generated in anoxic condition (Aru *et al.*, 2016).

Although many of the spoilage bacteria are proteolytic, they grow initially by utilizing the most readily available carbohydrates and nonprotein nitrogen. Glucose, lactic acid, and certain amino acids, followed by water-soluble proteins, are the precursors of metabolites that are responsible for meat spoilage. Moreover, concentrations of the precursors can influence the rate and extent of spoilage. It is the accumulation of microbial metabolites, such as aldehydes, ketones, esters, alcohols, organic acids, amines, and sulphur compounds, that determines the spoilage of meat (Ercolini *et al.*, 2011). As metabolites may be considered final downstream products of the genome and its interaction with the environment, the determination of metabolites might reveal interesting relationships between food consumption and possible variations in metabolic pathways (Castro-Puyana and Herrero, 2013). The data allow to reveals a clear discrimination between all tested conditions in this study: (i) the non-inoculated products at day 0 and 13, showing the natural evolution of the meat ecosystem according to the storage conditions; (ii) the inoculated and non-inoculated samples; (iii) the type of bacterium inoculated on the samples, reveals a clear discrimination on the metabolite profiles for *B. thermosphacta*, *P. fragi* and *L. gelidum*; and finally (iv) the packaging conditions, between MAP and FW packaging. In the next studies, these patterns will be more deeply studied. Finally, metabolites and their pathways could give important information about the spoilage capacity of inoculated bacteria, and particularly for *Pseudomas fragi* which seems to be of interest in our product.

5 CONCLUSIONS

These results support the use of NMR-based metabolomics as a valuable tool to provide information on minced pork meat spoilage and to follow intrinsically the evolution of the metabolomics pattern linked to a specific bacterium in a complex bacterial ecosystem. The data also suggest that NMR-based metabolomics is an efficient method to distinguish fingerprinting difference between samples, and metabolites can be possible biomarkers of spoilage products.

6 ACKNOWLEDGEMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

- Argyri, A. A., Mallouchos, A., Panagou, E. Z., & Nychas, G. J. E. (2015). The dynamics of the HS/SPME-GC/MS as a tool to assess the spoilage of minced beef stored under different packaging and temperature conditions. *International Journal of Food Microbiology*, *193*, 51-58.

- Aru, V., Pisano, M. B., Savorani, F., Engelsen, S. B., Cosentino, S., & Marincola ; C. F. (2016). Metabolomics analysis of shucked mussels' freshness. *Food Chemistry*, *205*, 58-65.
- Boffo, E. F., Tavares, L. A., Tobias, A. C. T., Ferreira, M. M. C., & Ferreira, A. G. (2012). Identification of components of Brazilian honeys by 1H NMR and classification of its botanical origin by chemometric methods. *Food Science Technology*, *49*, 55-63.
- Castejon, D., Garcia-Segura, J. M., Escudero, R., Herrara, A., & Cambero, M. I. (2015). Metabolomics of meat exudate: its potential to evaluate beet meat conservation and aging. *Analytica Chimica Acta*, *901*, 1-11. https://doi.org/10.1016/j.aca.2015.08.032. M. (2013). Metabolomics approaches based on mass spectrometry for food safety, quality and traceability. *Trends in Analytical Chemistry*, *52*, 74-87.
- Charlton, A. J., Farrington, W. H. H., & Brereton, P. (2002). Application of 1H NMR and multivariate statistics for screening complex mixtures: quality control and authenticity of instant coffee. *Journal of Agricultural and Food Chemistry*, *50*, 3098-3103.
- Chen, D. Ye, Y., Chen, J., & Yan, X. (2016). Evaluation of metabolomics profile of crab paste during fermentation. *Food Chemistry*, *192*, 886-892.
- Consonni, R., & Cagliani, L. R. (2008). Geographical characterization of polyfloral and acacia honeys by nuclear magnetic resonance and chemometrics. *Journal of Agricultural and Food Chemistry*, *56*, 6873-6880.
- Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A., Laghi, L., Mauriello, G., Guerzoni, M. E., & Villani, F. (2011). Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. *Applied and Environmental Microbiology*, *77*, 7372-7381.
- Jung, Y., Lee, J., Kwon, J., Lee, K. S., Ryu, D. H., & Hwang, G. S. (2010). Discrimination of the geographical origin of beef by 1H NMR-based metabolomics. *Journal of Agricultural and Food Chemistry*, *58*, 10458-10466. https://doi.org/10.1021/jf102194t.
- Lipinski, B., Hanson, C., Lomax, J., Kitinoja, L., Waite, R., & Searchinger, T. (2013). Reducing food loss and waste. World Resource Institute (June), 1-40.
- Mannina, L. Sobolev, A. P., & Viel, S. (2012). Liquid state 1H high field NMR in food analysis. *Progress in Nuclear Magnetic Resonance Spectroscopy*, *66*, 1-39.
- Miks-Krajnik, M., Yoon, Y. J., Ukuku, D. O., & Yuk, H. G. (2016). Identification and quantification of volatile chemical spoilage indexes associated with bacterial growth dynamics in aerobacilly stored chicken. *Journal of Food Science*, *81*, 2006-2014.
- Piras, C., Marincola, F. C., Savorani, F., Engelsen, S. B., Cosentino, S., Viale, S., & Pisano, M., B. (2013). A NMR metabolomics study of the ripening process of the Fiore Sardo cheese produced with autochothonous adjunct cultures. *Food Chemistry*, *141*, 2137-2147.
- Pothakos, V., Devlieghere, F., Villani, F., Björkroth, J., & Ercolini, D. (2015). Lactic acid bacteria and their controversial role in fresh meat spoilage. *Meat Science*, *109*, 66-74.
- Remenant, B., Jaffres, E., Dousset, X., Pilet, M.-F., & Zagorec, M. (2015). Bacterial spoilers of food: behavior, fitness and functional properties. *Food Microbiology*, *45*, 45-53.
- Ritota, M., Casciani, L., Failla, S., & Valentini, M. (2012). HRMAS-NMR spectroscopy and
- multivariate analysis meat characterization. *Meat Science*, *92*, 754-761.
- Saraoui, T., Cornet, J., Guillouet, E., Pilet, M.F., Chevalier, F., Joffraud, J.J., & Leroi, F. (2017). Improving simultaneously the quality and safety of cooked and peeled shrimp using a cocktail of bioprotective lactic acid bacteria. *International Journal of Food Microbiology*, *241*, 69-77.
- Yuan, Y., Zhao, Y., Yang, J., Jiang, Y., Lu, F., Jia, Y., & Yang, B. (2017). Metabolomic analyses of banana during postharvest senescence by 1H-high resolution-NMR. *Food Chemistry*, *218*, 406-412.
- Zanardi, E., Caligiani, A., Palla, L., Mariani, M., Ghidini, S., Di Ciccio, P. A., Palla, G., & Ianieri, A. (2015). Metabolic profiling by 1H NMR of ground beef irradiated at different irradiation doses. *Meat Science*, *103*, 83-89. https://doi.org/10.1016/j.meatsci.2015.01.005.

Development and Evaluation of W/O/W Emulsion Stabilized by Polysaccharide-Protein Based Interaction

Mishenki Rajapakse*, Anil K. Anal

Asian Institute of Technology, Khong Luang, Bangkok 12120, Thailand
*Corresponding Author (mishenki.raj1@gmail.com)

ABSTRACT

The potential of polysaccharide protein-based encapsulation systems to enhance the folic acid stability was evaluated. The optimal conditions to prepare stable W/O/W emulsions was determined by varying the concentration of polyglycerol polyricinoleate, dispersed phase volume fraction and the concentration of whey protein isolate and pectin. The emulsions were further characterized for their size, zeta potential and the polydispersity index. The particle size diameter varies from 1.36 µm to 2.51 µm. The zeta potential of the emulsions was recorded in the range of -31.33 mV to -33.70 mV. Moreover, polydispersity index results indicated that emulsions were monodispersed (PDI < 0.14). The behaviour of the emulsions was then studied in different digestion phases of the gastrointestinal tract. The ζ-potential of the freshly prepared double emulsion stabilized by both whey protein isolate and pectin and only whey protein isolate was recorded as -33.7 ± 0.7 mV and -41.7 ± 0.4 mV respectively. After the incubation period in mouth phase, the magnitude of the negative charge was reduced in emulsions containing both WPI and pectin, yet it was observed that the ζ-potential of the whey protein isolate stabilized emulsion was increased. A considerable change in the ζ-potential of the emulsion were observed after 2h of incubation period in the stomach phase. In the case of the whey protein isolate stabilized emulsions, the ζ-potential became more positive (18.80 ± 1.05 mV) than the emulsion which contained both whey protein isolate and pectin (3.08 ± 2.46 mV). In the simulated intestinal stage, the magnitude of the ζ-potential was observed to be more negative. The study revealed that whey protein and pectin interactions can enhance the stability of double emulsion in order to entrap hydrophilic bioactive compounds.

KEYWORDS: W/O/W Emulsion, Protein-Polysaccharide Interaction, Stability, Folic Acid, i*n vitro* Behaviour

1 INTRODUCTION

The design and developments of delivery systems are essential in food industry to protect, enhance bioavailability of active compounds and also to facilitate the controlled release. The selection of food matrices to serve as a delivery system is crucial because the system has to be food grade, efficient and cost effective (Tarun *et al*., 2017). The commercially available delivery systems to encapsulate hydrophilic bioactive compounds are minimal, such type of compunds are follows, water-soluble vitamins, nutraceuticals, colours, flavours, and preservatives (McClements D. J., 2015). During last decade colloidal systems are considered widely as a suitable delivery systems for nutrients in food products. Therefore nanostructure dispersions like microemulsions, nanoemulsions, double emulsions, liposomes, reverse micelles could be considered as an effective alternatives to traditional delivery systems like emulsions or microspheres, which were not suitable for many type of food products (Garti & Aserin, 2013).

A rising attention is being focused towards the microemulsion as an effective encapsulant in order to deliver nutraceuticals such as vitamins, functional oils through control release in the gastrointestinal tract and enhance the absorption (Xu *et al.,* 2016). Microemulsions can be considered as homogeneous mixtures of water, oil and relevant emulsifiers or surfactants, which are thermodynamically stable, optically isotropic and transparent due to their small droplet size (Flanagan & Singh, 2006). “Double emulsion” or “multiple emulsion” is known an emulsion of an emulsion. The applications of double emulsion to encapsulate bioactive compounds has triggered a growing interest in food industry recently. There are two main types of double emulsions, “water-in-oil-in-water

(W/O/W) and oil-in-water in- oil (O/W/O)" (Dickinson, 2011). Hydrophilic bioactive compounds can be entrapped within the inner aqueous phase of double emulsion which in turn enhance the protection, stability and bioavailability of a particular active compound (Dickinson, 2011). However, as known double emulsions are less thermodynamically stable, and also water-soluble vitamin can diffuse through the oil layers which on turn restricts the application of W/O/W multiple emulsions in food matrices (Garti & Aserin, 2013). Therefore, it's vital to increase the stability of double emulsions and in this study protein-polysaccharide based emulsifying system was used to enhance the stability of double emulsions.

As a nutrient compound vitamin plays an important role in human body. Vitamins regulate the growth and development of body as well as defends against various types of disease. Unfortunately, most of the vitamins are either produces in very minute amounts or not produced in body. Therefore, the vitamins have to be obtained from dietary supplements (Wildman, 2006). Vitamin deficiencies have caused many types of health problems around the world. The most critical vitamin deficiencies today are probably vitamin A, vitamin D and folic acid. Folate generally referred to vitamin B9 and mainly it has to be obtained from dietary sources. According to previous studies it has been found that low or inadequate folate may lead to some type of malfunctions and disorders (Iyer & Tomar., 2009). Mainly in the developing countries, folate deficiency is widely spread among people due to inadequate dietary intake of folate (McNulty, 2017). Thus, fortification of food with folic acid can be an effective solution for folate deficiency. However, folic acid does not have high stability towards the different type of harsh environmental conditions involved in food processing. Mainly it can be degraded while harvesting, storage, transportation, processing and cooking or preparation. More than half of original folate can be lost during food processing stages (FAO, 2001). These losses can directly influence the final nutritional quality of the food product. Therefore, they should be preserved from harmful environmental conditions. Encapsulation technique can be an effective way to protect folic acid and increase the bioavailability by incorporating into food matrices (Sanguansri & Augustin, 2006).

The main goal of this study was to prepare stable W/O/W emulsions considering the concentration of primary emulsifier, dispersed phase volume fraction and the concentration of polysaccharide-protein biopolymer solution and to study the *in vitro* behaviour of W/O/W emulsion in order to incorporate in food and beverage products.

2 MATERIAL AND METHODS

2.1 Materials

Folic acid was purchased from Xi'an Sgonek Biological Technology Co., Ltd (China). PGPR (Polyglycerol polyricinoleate) was purchased from Oleofine Organics (Thailand) Co., Ltd. Pectin was purchased from CTI & Science Co., Ltd (Thailand). Whey protein and alginate were purchased from a local chemical store. Sunflower oil was purchased from and from Big C Supercentre (Thailand). All chemical used for analysis were analytical grade.

2.2 Optimization of the Emulsion Condition

Emulsion condition have to be optimized in order to prepare stable emulsions. Firstly, the concentration of PGPR (1, 3, 5, 7, 9% w/w) varied to obtain stable primary emulsions. Secondly the effect of the dispersed phase volume fraction (DPVF) was studied (30: 70, 40: 50, 40: 60). The concentrations of WPI (2, 4, 6 w/w %) and the concentration of pectin (0.1, 0.3, 0.5, 0.7, 0.9 w/w %) was then varied to optimize the conditions required to prepare stable secondary emulsions. The required amount of folic acid was taken from a previous study conducted by Assadpour (2016). During the conditions, the emulsion stability, creaming index, microscopic structure, colour and the viscosity was measured.

Table 1: Optimization conditions of WPI and Pectin

Type	Concentration of WPI (% w/w)	Concentration of pectin (% w/w)
A_0	2	0
A_1	2	0.1
A_2	2	0.3
A_3	2	0.5
A_4	2	0.7
A_5	2	0.9
B_0	4	0
B_1	4	0.1
B_2	4	0.3
B_3	4	0.5
B_4	4	0.7
B_5	4	0.9
C_0	6	0
C_1	6	0.1
C_2	6	0.3
C_3	6	0.5
C_4	6	0.7
C_5	6	0.9

2.3 Preparation of WPI – Pectin Biopolymeric Solution

WPI powder and pectin powder was mixed together in distilled water to obtain required concentration as given in table 1. Then the mixture was homogenized at 500 rpm for 1 hour and the pH was adjusted using 1M NaOH. 0.02 % sodium azide was added as an antimicrobial agent. After homogenizing the solution was stored overnight at room temperature for complete hydration of biopolymers.

2.4 Determination of Properties of Emulsions

The emulsion stability was measured according to method described by Huang (2001) with some modification. 10 ml of emulsion was taken into a centrifuge tube and centrifuged at 2000 g for 5 min at 4^0C. Triplicates were conducted. The following equation was used to calculate emulsion stability.

$$\text{Emulsion stability (\%)} = \frac{H_2}{H_1} \times 100 \qquad (1)$$

Where H_1 is the initial height of the fresh emulsion and H_2 is the height of the emulsion after centrifugation.

The creaming index was determined by getting 5 ml of emulsion in a test tube and storing at cold room 4 °C for 24 hours. After that the cream the cream layer was measured.

$$\text{Creaming index (\%)} = \frac{H_C}{H_T} \times 100 \qquad (2)$$

Where H_C is the height of the cream layer and H_T is the height of the total emulsion.

2.3 Production of Primary Emulsion

W/O emulsions was prepared by according to the procedure described by Assadpour (2016). Firstly, the folic acid solution was prepared by mixing the required amount of folic acid in the phosphate buffer (3 mg in 1 ml of in phosphate buffer at pH = 7). The oil phase preparation was done by mixing 5% PGPR in sunflower oil and homogenized at 900 rpm before adding the inner aqueous phase. Then

the inner aqueous phase and the oil phase was mixed using a high-pressure homogenizer by adding aqueous phase drop wise.

2.4 Production of Double Emulsion

The preparation of $W_1/O/W/_2$ emulsions were conducted following the procedure described by the (Esfanjani, 2015). The previously prepared primary emulsion (W_1/O) was added gradually to the outer aqueous phase which formed from the WPI and pectin mixture. It was mixed using a high-pressure blender at 12,000 rpm for 2 min. The formed coarse emulsions were then further emulsified using a high-pressure homogenizer in three cycles and each cycle was carried out for 15 min.

2.5 Determination of Droplet Size and Droplet Charge

The particle size distributions and mean particle diameters (Z averages) of emulsions was measured by using a static light scattering instrument. Samples were diluted 100 times using deionized water to avoid the multiple scattering. The particle size data were reported as the intensity-weighted ("Z-average") mean particle diameter, while the particle charge data were reported as the z-potential.

2.6 Determination of *in vitro* Behaviour

The characteristics and the behaviour of emulsions during the digestion in gastrointestinal tract was evaluated using the emulsions stabilized with only WPI and emulsions with both WPI and pectin. The *in vitro* digestion was conducted mainly incubating the emulsions in simulated mouth, stomach and intestinal phases in a static in-vitro digestion model. After each incubation period the emulsions were for further testing. The temperature used during all the incubations was 37 ºC.

Mouth phase: Mouth phase was prepared by adding 0.022g of Musin, 0.0202 g of KCl and0.1594 g of NaCl. Then a phosphate buffer solution was added until the total weight become 100g. The pH was adjusted to 6.8 in the final solution. A 20 ml of fresh emulsion sample was mixed with 20 ml of prepared saliva solution and the pH was adjusted to 6.8 using 1 M NaOH. Then the mixture was incubated at 37 ºC for 10 while stirring at 100 rpm to resemble the mixing and the chewing process occurring in the mouth.

Gastric phase: The simulated gastric fluid (SGF) was prepared by mixing 1 g of NaCl, HCl 3.5 mL of HCl in 500 ml of phosphate buffer. Then 1.2 g of pepsin was added. The pH was set to 1.2 using 1 M HCl. The emulsion sample after the incubation at mouth phase was used, therefore 20 ml of emulsions from the mouth phase was mixed with 20 ml of simulated gastric solution. Then the mixture was incubated at 37 ºC for 10 while stirring at 100 rpm.

Intestinal phase: A fresh simulated intestinal fluid (SIF) was formulated by dissolving 0.02g of 39mM K_2HPO_4, 0.26 g of 150 mM NaCl and 0.01 g of 30 mM $CaCl_2$ in 30 ml of phosphate buffer solution. The pH was adjusted to 7.5. Bile salts (5 mg/ml) and pancreatin (1.6mg/ml) were further added. The gastric digested emulsion also known as the chyme sample was added to the SIF solution (30 mL)

During the in-vitro digestion process, the emulsion samples were collected after each incubation period and characterized for their particle size, ζ-potential and morphology.

3 RESULTS AND DISCUSSION

3.1 Effects of Concentrations of PGPR on the Stability of Primary Emulsions

The different concentrations of PGPR (1, 3, 5, 7 and 9% w/w) were used to prepare stable emulsions. The PGPR is considered as one of the most efficient oligomeric emulsifiers. Creaming index of the primary emulsions were calculated and the results were given in the figure1. The previous studies have also found that the higher concentration of PGPR gives the more stable emulsions. The creaming index of 5%, 7% and 9% PGPR was lower than the creaming index of 1% and 3% PGPR. The highest creaming index was observed from 1% PGPR and the lowest was from the 10% PGPR. Although the creaming index of 10% PGPR was lower among the other concentrations, the 5% PGPR was selected

as the most suitable concentration to prepare W/O/W emulsions because the greater concentration than 5% might add off flavours and unpleasant tastes to food products (Muschiolik, 2006).

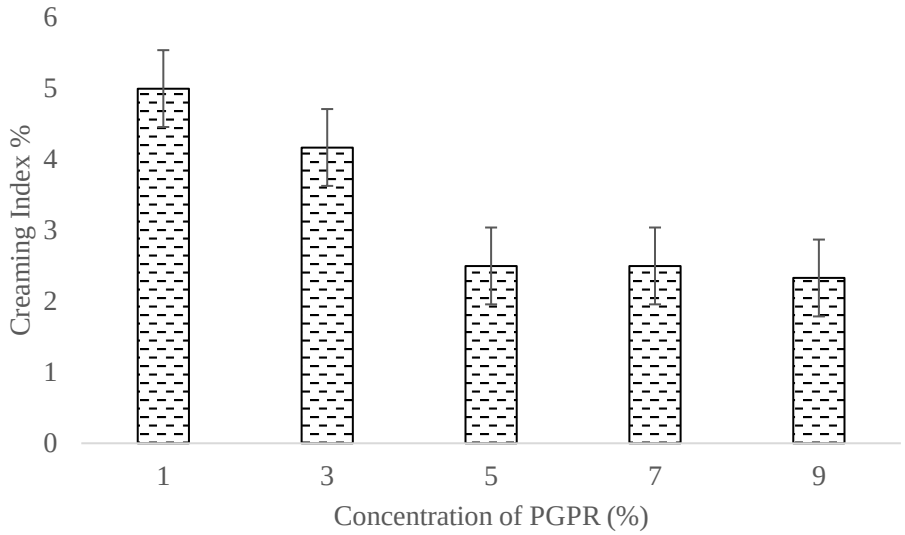


Figure 1: Effect of PGPR concentration on the creaming index of emulsions

3.2 Effect of Dispersed Phase Volume Fraction on the Stability of Double Emulsions

The volume fraction of the dispersed phase (W1/O) to the continuous phase (W2) was studied using three different ratios (30: 70, 40: 60, and 50: 50). According to the obtained results, it was determined that the volume fraction of 50:50 would give stable double emulsions than the other volume fractions. As per to microscopic images of the respective emulsions (images are not shown), the droplet size if the emulsions were decreased with the increasing volume fraction. These results can be supported by the findings of the Merve and others (2016), where it has claimed that the composition of the primary emulsion incresed, the particle size of the droplet decreased. In contrast to these observations, it has been reported that increasing DPVF can results bigger droplet sizes which in sequence influence the stability of emulsions (Jafari et al., 2008).

Xiao and others (2017) prepared double emulsion using the volume ratio of W1/O: W2 = 50:50 to encapsulate anthocyanin using 1.5 wt.% kafirin nanoparticles from sorghum grain, which results stable double emulsions with 85% encapsulation efficiency.

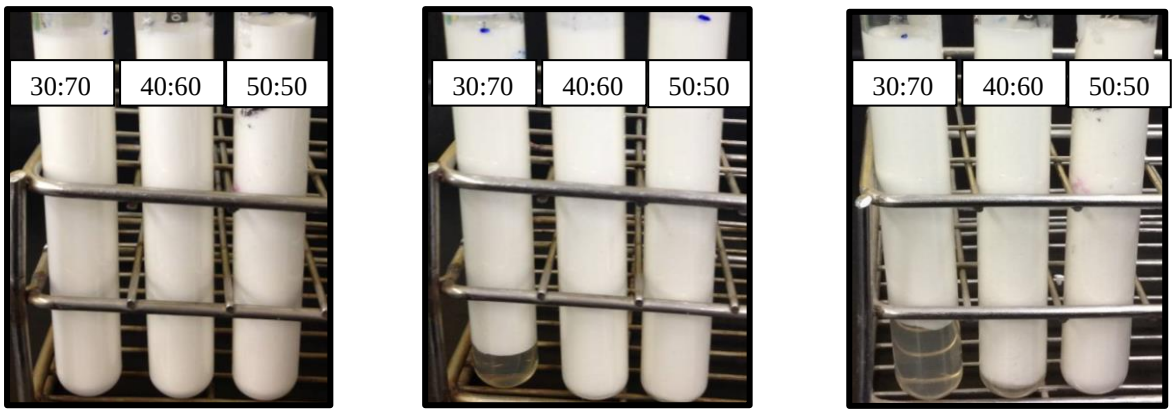


Figure 2 Physical stability of double emulsion on different volume fraction at day 0, 1 and 2

3.3 Effect of Concentration of Whey Protein Isolate and Pectin on the Stability of Double Emulsions

Whey protein isolate (WPI) and the pectin combination was used as the secondary emulsifiers of the double emulsion. WPI and the pectin was mixed together in distilled water pH was adjusted to 7. Then the biopolymeric solution was kept for 24 h in cold temperature (4 °C).

Table 2: Different concentration of WPI and pectin used for optimization of emulsions

Emulsion type	Concentration of WPI (% w/w)	Concentration of pectin (% w/w)
A_0	2	0
A_1	2	0.1
A_2	2	0.3
A_3	2	0.5
A_4	2	0.7
A_5	2	0.9
B_0	4	0
B_1	4	0.1
B_2	4	0.3
B_3	4	0.5
B_4	4	0.7
B_5	4	0.9
C_0	6	0
C_1	6	0.1
C_2	6	0.3
C_3	6	0.5
C_4	6	0.7
C_5	6	0.9

The physical stability of the double emulsions, microscopic structure, creaming index, viscosity and colour was determined in order to get the optimized conditions to prepare stable double emulsions (Data of colour, viscosity and microscopic images are not shown).

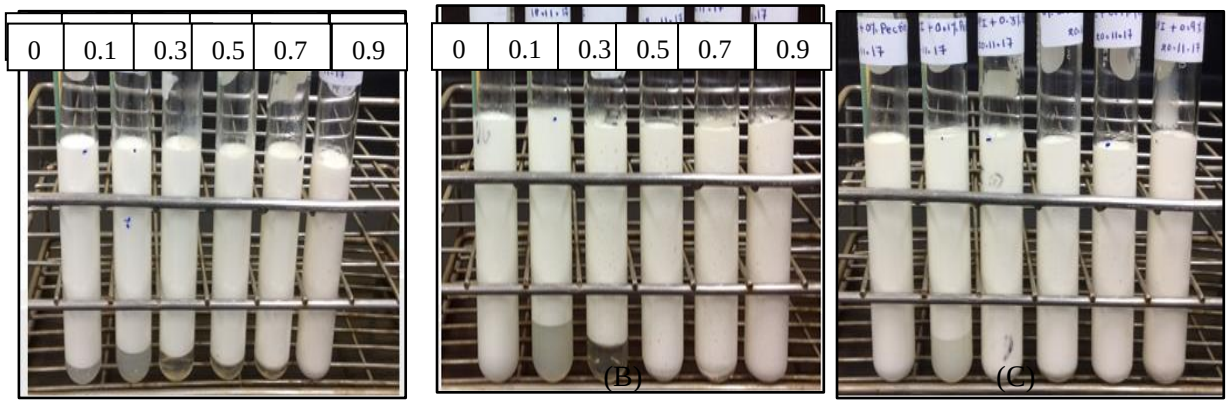


Figure 3 Effect of pectin concentration (w/w %) on the physical stability of the emulsions
(A) 2% WPI **(B)** 4% WPI **(C)** 6% WPI

The physical stability of the double emulsion is given in the figure 3. When focusing about the emulsions made up of WPI alone, the physical stability of emulsion was increased with the increasing concentration of the WPI. Therefore, the highest physical stability was obtained from the emulsions prepared with 6% WPI. Due to the high amount of whey protein, it can sufficiently cover the surface of oil droplets which in turn limits the coalescence or the aggregation of droplets. When the

concentration of the WPI is less, it might be not sufficient or adequate enough to coat the oil droplets and act as a thick layer around the droplet (Mcclements et al., 1993).

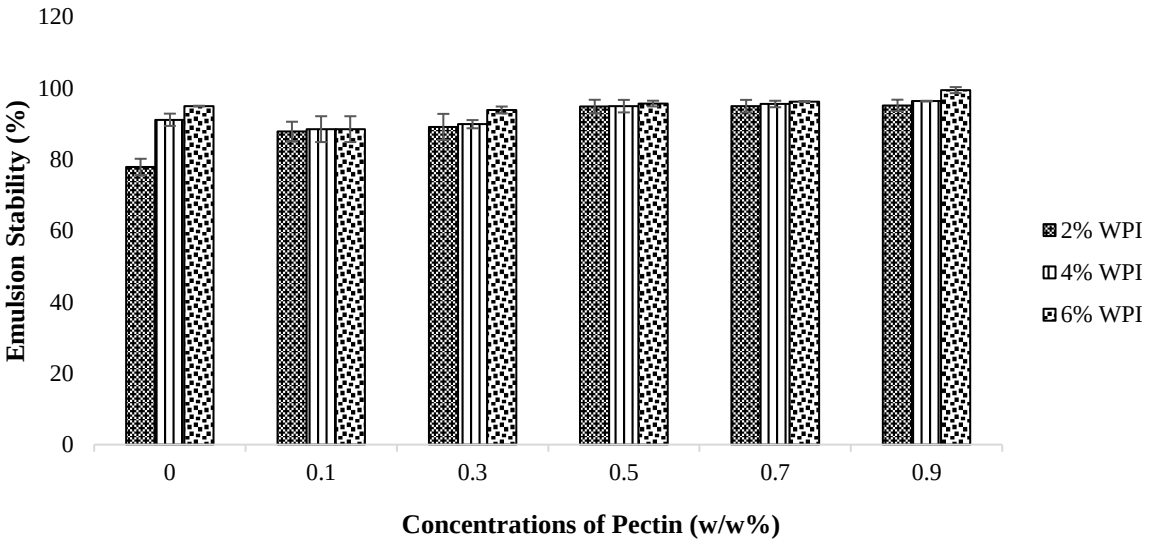


Figure 4: Effect of concentration of pectin (w/w%) on the emulsion stability

The effect of pectin concentration was studied using five different concentration of pectin (0.1, 0.3, 0.5, 0.7 and 0.9% w/w). The emulsions stabilized with the 2% WPI were susceptible to phase separation even with the high concentration of pectin. The stability of A_0 and A_1 emulsion were significantly lower ($p < 0.05$) than the stability of the emulsions with higher concentrations of pectin. When the concentration of WPI is less, a high amount of pectin may require to obtain stable emulsions. In the case of 4% WPI, the physical stability of emulsion decreased with the low concentrations of pectin (0.1, 0.3% w/w) than the WPI alone condition as shown in figure 4. Though with high concentrations of pectin (0.5, 0.7, 0.9% w/w) the stability of emulsion increased. The emulsions stabilized with 6% WPI showed the highest stability with the added pectin. Only the C_1 emulsion showed a phase separation. Similar observation has been revealed by Einhorn-Stoll and colleagues (1996), when a small amoun of pectin (around 0.1%) was added the stability of the emulsion decreased slightly, whereas a higher concentration more than 0.2% had decreased the phase separation of the emulsion and increased the stability. When the concentration of pectin is low, the number of electrostatic interaction are not adequate enough to form strong interactions, causing the destabilization of the emulsions. According to Mao and others (2013), a pectin concentration of 0.8% had been resulted the highest stability of multilayer o/w emulsion stabilized with whey protein isolate.

Prior researchers have emphasized that, when there is a protein which adsorbs to a fluid surface and if also there is a polysaccharide present, mainly there are three possibilities that could take place. The polysaccharide will compete with the protein to adsorb to the interface due to its surface-active nature. Secondly, the polysaccharide will make complexes with the adsorbed protein. Lastly, polysaccharide concentrates the adsorbed protein by a depletion mechanism (Patino & Pilosof, 2011).

3.4 Characterization of Emulsions

3.4.1 Particle Size, Zeta Potential and PDI

It can be clearly observed that when the WPI concentration increasing, the droplet size was decreasing. The highest droplet size of 2.51 ± 0.20 µm was resulted from B_4 emulsion. The lowest droplet size was obtained from C_5 emulsion, which was recorded as 1.36 ± 0.07. Murillo-Martínez and others (2011) has used WPI and pectin to stabilize double emusions and the droplet size was found to be 2.47 µm. The partial size of the 4% WPI stabilized emulsions were significantly ($p < 0.05$) higher than the particle size of the 6% WPI stabilized emulsions. The increase in protein content can create

more thick and steady coats on the oil droplets causing steric repulsions, and also it may lower the density difference between the water phase and the oil droplet. Hence decreasing the creaming and coalescence. Moving in to the effect of pectin concentration, it showed a significance ($p < 0.05$) increment in the particle size of the B_4 and B_5 emulsion. Though there was no significance difference between the particle sizes of 6% WPI with the increasing concentration of pectin (Murillo-Martínez *et al.*, 2011)

The ζ-potential of the emulsions was in the range of -31 mv to -34 mV. It is known that a ζ-potential greater than 30 mV or less than -30 mV can stabilize double emulsions (Wang et al, 2017). The highest ζ-potential of -33.70± 0.70 mV was recorded in the C_5 emulsion while the B_5 emulsion showed lowest zeta potential of -31.33 ± 0.58 mV. The ζ-potential has become more negative with the increasing concentration of WPI. Because when the amount of adsorption of WPI increases, it will increase the negative of the ζ-potential. Due to the increasing concentration of WPI, the availability of WPI to form an interfacial layer around oil droplets will also increase which in turn increase the negative charge of the system (Tcholakova *et al.*, 2003).

The polydispersity index (PDI) can be considered as a dimensionless measure which is used to determine a particle size distribution. PDI values between 0.03 and 0.06 are identified as monodisperse, while the values between 0.1 and 0.2 as denoted as narrowly distributed and between 0.25-0.5 as broadly distributed and value above 0.5 indicated extremely broad size distribution that cannot be described by means of PDI (Muller, 1996). Except for the emulsion with 4% WPI + 0.7% pectin, all the other emulsion types showed a PDI value less than 0.1 indicating a monodisperse behaviour. Although there was no any significant ($p < 0.05$) effect from the concentration of pectin on the PDI, when the biopolymer concentration was increasing the PDI value got decreased. This possibly due to the higher viscosity of the continuous phase of the emulsion which limits the movement of the droplets.

Table 3: Particle size, Zeta potential and the PDI of the emulsions

Emulsion Type	Particle size (µm)	Zeta potential (mV)	PDI
B_4	2.51 ± 0.20 ^{c}	-31.73 ± 0.47 ^{a}	0.13 ± 0.04 ^{b}
B_5	1.74 ± 0.05 ^{b}	-31.33 ± 0.58 ^{a}	0.07 ± 0.06 $^{a\,b}$
C_4	1.55 ± 0.04 $^{b\,a}$	-32.40 ± 0.96 $^{a\,b}$	0.03 ± 0.02 ^{a}
C_5	1.36 ± 0.07 ^{a}	-33.70 ± 0.70 ^{b}	0.03 ± 0.02 ^{a}

B_4 - 4% WPI + 0.7% Pectin, B_5- 4% WPI + 0.9% Pectin, C_4 - 6% WPI + 0.7% Pectin, C_5 - 6% WPI + 0.9% Pectin

3.5 *In vitro* Behaviour of Emulsion

3.5.1 Zeta Potential

The electrostatic interactions and their influence on the stability of emulsions can be measured in terms of zeta potential (ζ -potential) (McClements & Decker, 2009). Therefore ζ-potential can be used as a measurement to identify the variations in interfacial composition of the emulsion droplets during in each stage of the gastro intestinal tract.

The ζ-potential of the freshly prepared double emulsion stabilized by WPI and pectin was recorded as -33.7 ± 0.7 mV. The ζ-potential of the WPI stabilized emulsion was recorded as the -41.7 ± 0.4 mV. After the incubation period in mouth phase, the magnitude of the negative charge was reduced in emulsions containing both WPI and pectin, which might be due to the mineral ions in the simulated saliva solution that increases the ionic strength. Another possible cause could be the adsorption of charged mucin molecules to the droplet surface and affecting the charge characteristics. Yet it was

observed that the ζ-potential of the WPI stabilized emulsion was increased up to -44.9 ± 1.36 mV. It is known that negatively charged emulsion droplets may be subjected to reversible bridging flocculation in the oral phase (Sarkar, 2012).

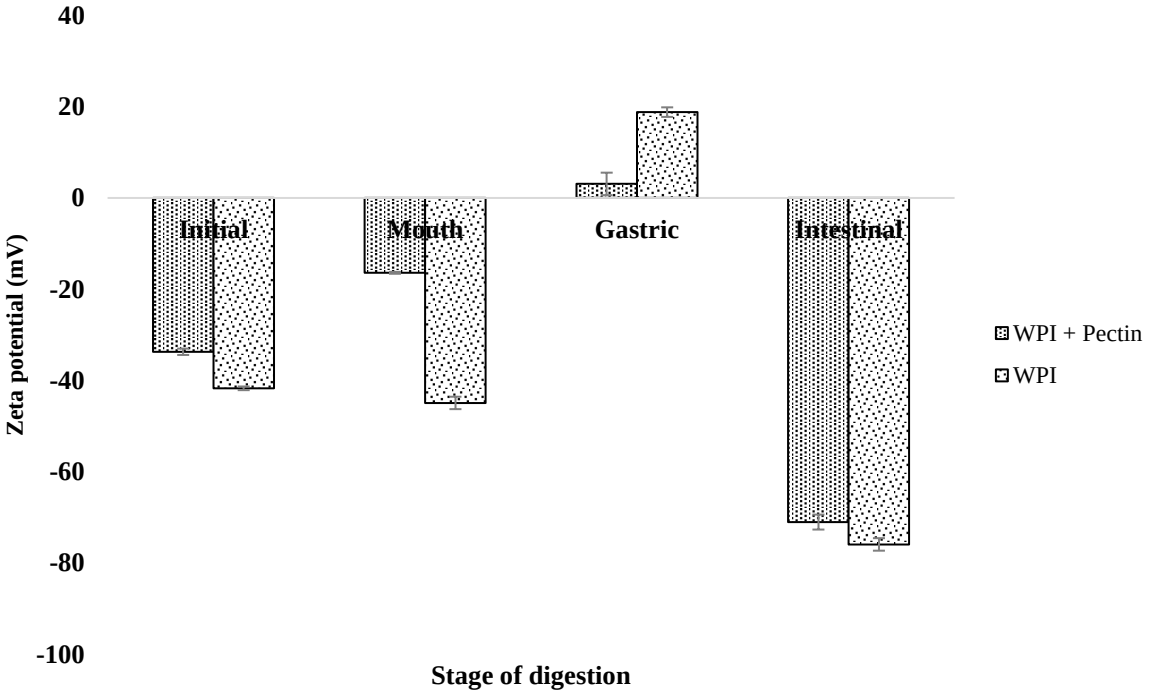


Figure 5: Zeta potentials of emulsion at different digestion stages

After the mouth phase, the emulsions were transferred to the gastric phase. A considerable change in the ζ-potential of the emulsion were observed after 2h of incubation period. In the case of the WPI stabilized emulsions, the ζ-potential became more positive (18.80 ± 1.05 mV) than the emulsion which contained both WPI and pectin (3.08 ± 2.46 mV). These variation in the ζ-potential can be described by phenomena of the isoelectric point where the protein molecules become positive in the pH values less than the isoelectric point of the protein. Previous studies have also been claimed that a positive charge of the emulsions after exposing to the gastric phase (McClements & Decker, 2009).

The emulsions from the stomach phase then introduced into the simulated intestinal stage. The magnitude of the ζ-potential was observed to be more negative. The more negativity of the ζ-potential can be explained based on the pH value where the protein and polysaccharide carry more negative charge above their isoelectric point. As shown in the figure 5, the WPI stabilized emulsions did not reached to the initial value which can be due to the digestion of some whey protein by the enzymatic activity of the pepsin. The recorded ζ-potential of the WPI stabilized emulsion was 75.9 ± 1.37 mV. The droplet charge in emulsions containing both WPI and pectin was also more negative (- 71 ± 1.64 mV) than the initial value. This can be explained by the fact that pectin would interrupt the enzymatic activity of pepsin to digest protein, by the interaction formed with the protein thus preventing the dislocation of whey protein.

3.6 Droplet Size Distribution

The initial diameter of the WPI + pectin emulsion was 1.36 ± 0.07 µm while the particle size of the only WPI stabilized emulsion was 4.08 ± 0.09 µm. The initial PDI of the emulsion of WPI and WPI+ pectin was 0.49 ± 0.26 and 0.03 ± 0.02. As the initial PDI of WPI +pectin emulsion was less than 0.1, it can be suggested that pectin did not promote any droplet aggregation.

When the fresh emulsions were incubated in the mouth phase, there was no significance ($p < 0.05$) difference in the PDI of the emulsion, yet the particle size varied significantly ($p > 0.05$) comparatively to the initial stage. After incubation in the simulated stomach phase, the particle size and the PDI of the WPI + pectin emulsion increased significantly ($p < 0.05$). In opposition to these observations, only WPI stabilized emulsion did not show any significance difference ($p > 0.05$) either in particle size or the PDI.

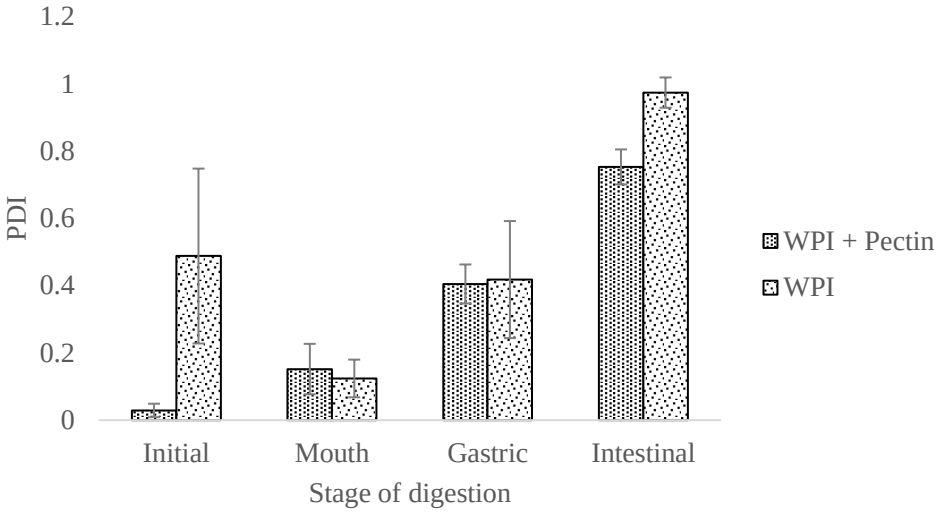


Figure 6: Polydispersity indexes of emulsion at different digestion stages

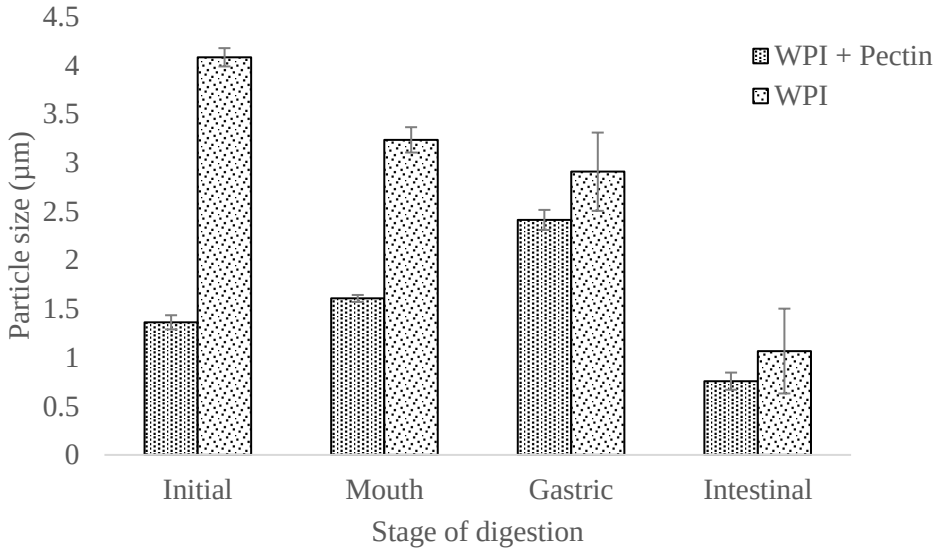


Figure 7: Particle sizes of emulsions at different digestion stages

The droplet size of the emulsion was varied drastically after the incubation period of the intestinal phase. The droplet size of the whey protein stabilized emulsion was recorded as 0.76 ± 0.08 µm while the emulsions with both whey protein showed a higher particle size (1.06 ± 0.04 µm). This might be due to the more stable interaction between whey protein and pectin which in turn enhance the stability of the emulsions.

4. CONCLUSION

The main object of this study was to design and develop a protein-polysaccharide based encapsulation system to entrap hydrophilic bioactive compounds. Folic acid was encapsulated in W/O/W double emulsion stabilized by whey protein isolate and pectin. By varying the optimization

conditions, it was found that 5% PGPR and the volume fraction of 50:50 would result in stable double emulsion system. Additionally, the emulsions stabilized with 6% WPI showed the highest stability with the added pectin. Thus, four types of emulsions were chosen for further characterization (4% WPI+0.7% pectin, 4% WPI+0.9% pectin and 6%WPI + 0.7% pectin, 6% WPI + 0.9% pectin). The particle size diameter was obtained in the range of 1.36 µm to 2.51 µm, zeta potential of the emulsions was recorded in the range of -31.33 mV to -33.70 mV while PDI results indicated that emulsions were monodispersed (PDI < 0.14). It was observed that when the WPI concentration increasing, the droplet size was decreasing. The partial size of the 4% WPI stabilized emulsions were significantly ($p < 0.05$) high than the particle size of the 6% WPI stabilized emulsions. After the incubation period in mouth phase, the magnitude of the negative charge was reduced in emulsions containing both WPI and pectin, yet it was observed that the ζ-potential of the whey protein isolate stabilized emulsion was increased suggesting that the negatively charged emulsion droplets may be subjected to reversible bridging flocculation in the oral phase. A considerable change in the ζ-potential of the emulsion were observed after 2h of incubation period in stomach phase. In the case of the WPI stabilized emulsions, the ζ-potential became more positive due to the phenomena of isoelectric point. After introducing the emulsion to the intestinal phase the magnitude of the ζ-potential was observed to be more negative. The droplet size of the emulsion was varied drastically after the incubation period of the intestinal phase, which interprets more stable interaction between whey protein and pectin. This study demonstrated that more stable W/O/W micro emulsion could be made successfully by using WPI-Pectin combination as the secondary emulsifier.

5 ACKNOWLEDGEMENT

AIT Fellowship offered to Mishenki Rajapakse by the Asian Institute of Technology, Thailand is thankfully acknowledged.

REFERENCES

- Dickinson, E. (2011). Double Emulsions Stabilized by Food Biopolymers. In *Food Biophysics* (pp. 1-11). Springer US.
- Einhorn-Stoll, N. U. (1996). Modified pectins in whey protein emulsions. *Molecular Nutrtion Food Research*, 60-67.
- Elham Assadpour, Y. M.-M. (2016). Optimization of Folic Acid Nano-emulsification and Encapsulation by Maltodextrin-Whey Protein Double Emulsions. *International Journal of Biological Macromolecules*, 197-207.
- Esfanjani, A. F. (2015). Nano-encapsulation of saffron extract through double-layered multiple emulsions of pectin and whey protein concentrate. *Journal of Food Engineering*, 149–155.
- FAO. (2001). *Human Vitamin and Mineral Requirements.* doi:http://www.fao.org/3/a-y2809e.pdf
- Flanagan, J., & Singh, H. (2006). Microemulsions: A potential delivery system for bioactives in food. *Critical Reviews in Food Science and Nutrition.*, 221-237.
- Garti, N., & Aserin, A. (2013). Double Emulsions. In T. Tadros, *Encyclopedia of Colloid and Interface Science* (pp. 303-337). Springer, Berlin, Heidelberg.
- Huang, X. K. (2001). Hydrocolloids in emulsions: particle size distribution and interfacial activity. *Food Hydrocolloids*, 533-542.
- Iyer, R., & Tomar., S. (2009). *Folate: a functional food constituent.* Journal of Food Science.
- Jie Xiao, X. L. (2017). Double emulsion derived from kafirin nanoparticles stabilized Pickering emulsion: fabrication, microstructure, stability and in vitro digestion profile. . *Food Hydrocolloid*, 230-238
- Mao, L. R. (2013). Volatile Release from Whey Protein Isolate—Pectin Multilayer Stabilized Emulsions: Effect of pH, Salt, and Artificial Salivas. *Journal of Agricultural and Food Chemistry*, 6231–6239.
- Mcclements, D. J. (1993). Disulfide bond formation affects stability of whey protein isolate emulsions. *Journal of Food Science*, 1036–1039.

- McClements, D. J. (2015). *Nanoparticle- and Microparticle-Based Delivery Systems.* Boca Raton: CRC Press. Retrieved from https://www.taylorfrancis.com/books/9781482233162
- McClements, D. J., & Decker, E. (2009). *Designing Functional Foods.* USA: Woodhead Publishing.
  Retrieved from https://www.elsevier.com/books/designing-functional-foods/mcclements/978-1-84569-432-6
- McNulty, H. (2017). The Role of Food Fortification: The Example of Folic Acid. In A. D. Hans Konrad Biesalski, *Sustainable Nutrition in a Changing World* (pp. 371-388). Springer, Cham.
- Merve Yildirim, G. S. (2016). The effects of emulsifier type, phase ratio, and homogenization methods on stability of the double emulsion. *Journal of Dispersion Science and Technology*, 807-814.
- Muller, R. H. (1996). Particle Charge and Surface Hydrophobicity of Colloidal Drug Carriers. *Targeting of Drugs With Synthetic Systems*, 239-263.
- Murillo-Martínez, M. P.-I.-C.-F.-C. (2011). Designing W1/O/W2 double emulsions stabilized by proteinepolysaccharide complexes for producing edible films: Rheological, mechanical and water vapour properties. *Food Hydrocolloids*, 577 - 585.
- Muschiolik, G. S. (2006). Multipleemulsions – preparation and stability. *Proceeding of the Thirteenth World Congress of Food Science & Technology*. doi:10.1051/IUFoST:20060043
- Patino, J. M., & Pilosof, A. (2011). Proteinepolysaccharide interactions at fluid interfaces. *Food Hydrocolloids*, 1925-1937.
- Sanguansri, P., & Augustin, M. A. (2006). *Nanoscale materials developmentea food industry perspective.* Australia: Trends in Food Science & Technology.
- Sarkar, A. &. (2012). Oral Behaviour of Food Emulsions. *Food Oral Processing: Fundamentals of Eating and Sensory Perception.*, 111–137.
- Seid Mahdi Jafari, E. A. (2008). Re-coalescence of emulsion droplets during high-energy emulsification. *Food Hydrocolloids*, 1191-1202.
- Slavka Tcholakova, N. D. (2003). Interrelation between Drop Size and Protein Adsorption at Various Emulsification Conditions. *Langmuir*, 5640–5649.
- Tarun, G. S. (2017). Colloidal systems: an excellent carrier for nutrient delivery. *Nutrient Delivery, Nanotechnology in the Agri-Food Industry*, 681-712.
- Wang, J. A. (2017). Formulation of water-in-oil-in-water (W/O/W) emulsions containing trans-resveratrol. *The Royal Society of Chemistry.*, 35917–35927.
- Wildman, R. E. (2006). *Handbook of nutraceuticals and functional foods.* CRC Press. Retrieved from https://www.taylorfrancis.com/books/e/9781420006186
- Zhenbo Xu, J. J. (2016). Co-surfactant free microemulsions: Preparation, characterization and stability evaluation for food application. *Food Chemistry*, 194-200.

Box-Behnken Design Based Mango (Mangifera indica) Wine Preparation, Screening of Phytochemicals and Antioxidant Activity by DPPH Radical Scavenging Assay

Win Nandar Thet*, Theint Theint Hlaing, Su Lwin Htike

Department of Chemical Enginnering,Yangon Technological University, Yangon, Myanmar [Burma]
*Corresponding author (winnandarthet@gmail.com)

ABSTRACT

Preparation of mango wine was optimized by Box-Behnken design. Variable factors were water to mango pulp ratio, yeast concentration and sugar concentration. Based on ANOVA analysis, $R^2 > 0.99$ and $P < 0.05$, the optimum conditions were water to mango pulp ratio (2:1), yeast concentration 2.5 g/L and initial sugar concentration 22%. The mean values of alcohol content, pH and total soluble solids were 7.4%, 3.91 and 7 (ºBx). The obtained wine was screened for phytochemicals (bioactive substances) such as alkaloids, carbohydrate, glycosides, phenolic compounds, α -amino acids, saponins, tannin, flavonoids, steroids, terpenoids, reducing sugar, starch and cyanogenic glycosides. Most of the phytochemicals were present in mango wine except saponin, tannin and cyanogenic glycosides. Then the antioxidant property was investigated with 2, 2-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay in vitro. IC_{50} was calculated and the value was < 50 ppm. The above findings showed that the mango wine prepared by using Box-Behnken design had bioactive compounds and antioxidant properties which gave valuable medicinal effects.

KEYWORDS: ANOVA, Antioxidant, Box-Behnken design, DPPH

1 INTRODUCTION

Mango (*Mangifera indica* L.) is the most economically important fruit in the Anacardiaceace (Cashew or poison ivy family). Mango is the most important foodstuff for inhabitants of the tropics after the banana. There are 150 cultivars of mangoes produced around the world. It is native to Myanmar.(Medina & García, 2002)

Among the horticultural fruits, mango plays a central role as fruit crop in Myanmar. The total mango production area was 70,084 hectares in 2010-2011. It occupied 11.85% of total horticultural area. The majority of mango is consumed as fresh dessert fruit in the ripening stage, as salad in the immature stage, i.e. with the endocarps not yet hardened, and as various types of pickles and dried preserves. In Myanmar, there are about 300 varieties and 20 kinds of mango species. Among them, there are only a few cultivars such as 'Sein Ta Lone', 'Yin Kwe', 'Shwe Hin Thar', and 'Mya Kyauk' which are exportable quality including high sweetness level . Other cultivars, namely 'Aung Din', 'Khaung Kyoe', 'Ma Chit Su', 'Pan Swal', 'Sein Myet Kone', 'Byoke', etc., are produced as fresh fruit and value added products for local consumption. Fresh mangoes are mainly exported to China by border trade and to Singapore by overseas trade.(Myat, Canavari, & Darnhofer, 2013)Production of wine from mango is one of the alternative ways to use and convert surplus production into a valuable product.

Wine is a product of alcoholic fermentation of grape or any other fruit juice with a good proportion of sugar. In general, the main raw material for the wine production is grape, but the suitability of different fruits for the wine-making has been investigated significantly in the previous decade. Highly acceptable fruit wines are obtained from apple, mango, banana, peach, raspberry , blackberry , plum etc (Miljić & Puškaš, 2014). Wine is a transformation of juice by yeast during fermentation involving a series of biochemical reactions. The biological process of wine-making is the result of a series of biochemical transformations. The series of biological transformations are brought about by the action of several enzymes from different microorganisms, especially yeasts. The *Saccharomyces* species, especially *S. cerevisiae*, is the dominant yeast utilised in commercial wine production today. (Jie, 2014) Yeasts are responsible for the principal part of the process, alcoholic fermentation. Lactic acid bacteria are responsible for a secondary process, known as malolactic

fermentation.Wine represents a rich source of polyphenols like anthocyanins, catechins, proanthocyanidins, flavonols, stilbenes, etc. Most of the compounds possess antioxidant properties that may protect against cardiovascular disease. Fruit wines with a higher concentration of total phenolics and anthocyanins and with a higher antioxidant capacity exhibited the strongest cytotoxicity towards cancer cells Fruit wines inhibited the growth of human cancer cells *in vitro* in a dose--dependent manner with differing susceptibility among tested cancer cells.(Ljevar,2016)

'Phyto' is the Greek word for plant. Phytochemicals could refer to every naturally-occuring chemical in plants. In practice, nutritionists use phytochemicals to refer naturally occurring compounds of plants that have physiologically effects in humans. There are many 'families' of phytochemicals and they help the human body in a variety of ways.(Kumar,2009)

Phytochemicals (Bioactive non-nutrient plant compound) in fruits, vegetables, grains, and other plant foods have been linked to reductions in the risk of major chronic diseases. It is estimated that more than 5000 phytochemicals have been identified but a large percentage still remain unknown and need to be identified before their health benefits are fully understood.

Under environmental stress, phenolic compounds increase and play a vital role in plant survival. Recent awareness of the role of antioxidants plays in the promotion of health, due to their ability to act as chemoprotective agents. Polyphenols contribute to colour and other sensorial characteristics of wines such as bitterness and astringency.

Bioactive compounds can protect against diseases via several mechanisms, but it is believed that the antioxidant activity is extremely important for protection against diseases related to oxidative stress. Mango wine is capable of preventing lipid peroxidation through scavenging free radicals and its antioxidant activities could be due to the synergic effect of carotenoids, glutathione and polyphenols.

The aim of this research is to investigate the influence of initial sugar concentration, yeast concentration and water ratio on mango wine and to find the optimum conditions in mango wine product by the use of RSM and Box-Behnken design. In view of the foregoing and importance of phenolic compounds as antioxidants in wine, the present investigation has been undertaken to determine the phytochemicals (bioactive substances) and to evaluate antioxidant properties of mango wine.

2 MATERIALS AND METHODS

2.1 Sample Collection

Mature, sound and healthy mango fruits (Yin Kwe cultivars) were procured from Thiri Mingalar Market.

2.2 Fruit Juice Preparation

Ripe mangoes were sorted, washed, peeled, deseeded and homogenized with blender. Then the juice was pasteurised at 65ºC for 10 minutes and cooled.

2.3 Fermentation of Fruit Juice

The juice was mixed with different ratios of mango and water (1:1, 1:2, 1:3, 1:4), different concentrations of Baker's yeast (1, 2, 3, 4 g/L) and different sugar concentrations (20, 21, 22, 23 %) according to Box-Behnken design as shown in Table 1 and Table 2. Fermentation was carried out for 14 days.

2.4 Experimental Design

Box-Behnken design was used for optimization of mango wine formulation. The parameters studied as independent variables were initial sugar concentration, yeast concentration and water ratio. The parameters selected were analysed with different levels (low and high coded as -1 and 1) levels.

2.4 Determination of pH

The pH was measured by the AOAC Official Methods of Analysis, Method No. 960.19, 16th ed, 1995. The pH meter used was (OHAUS pH meter, STARTER 3100, New Jersy, USA).

2.5 Determination of Total Soluble Solids (TSS)

The total soluble solids was determined according to the AOAC Official Methods of Analysis, Method No. 962.12, 16th ed., 1995 with the hand refractometer (TOSHNIWAL ROCKET, 0-30, S. No. 526, Tamil Nadu, India). The readings were expressed in ºBx.

2.6 Determination of Alcohol Content

By the use of hydrometer, alcohol meter (MC, 0-50, 20℃, China), the alcohol content was measured.

2.7 Preliminary phytochemical screening of wine

Phytochemical such as alkaloids, glycosides, flavonoids, tannins, polyphenolic compounds, alpha amino acids, saponins, steroids/ terpenoid, carbohydrates, protein, reducing sugar, starch were examined by test tube method.

2.8 Determination of antioxidant activity by DPPH radical scavenging assay

Different concentrations of wine samples were tested using gallic acid as a standard for calibration and the absorbance was measured at 517 nm with UV-Vis spectrophotometer.

3 RESULTS AND DISCUSSION

Table 1 represented the values of factors in Box-Behnken design with different levels and coded as +1, 0 and -1.

Table 1: Value of Factors in Box-Behnken Design

Level	Sugar (%)	Yeast (g)	Water (mL)	X_1	X_2	X_3
High	23	2	400	+1	+1	+1
Center	21.5	1.25	325	0	0	0
Low	20	0.5	250	-1	-1	-1

A total 15 combinations were prepared and the responses such as alcohol (%) and TSS were shown in Table 2.

Table 2: Box-Behnken Experimental Design for Mango Wine Formulation

Run order	X_1	X_2	X_3	Alcohol (%)	TSS (ºBx)
1	-1	- 1	0	5	6
2	+1	- 1	0	9	10
3	- 1	+1	0	6	6
4	+1	+1	0	10	10
5	-1	0	-1	6	6
6	+1	0	-1	9	10
7	-1	0	+1	6	6
8	+1	0	+1	10	10
9	0	-1	-1	6	7
10	0	+1	-1	7	6
11	0	-1	+1	7	7
12	0	+1	+1	7	7

13	0	0	0	7	7
14	0	0	0	7	7
15	0	0	0	7	7

Table 3: Second-order Polynomial Models for Investigated Responses

Parameter	Equation	R^2
Alcohol (%)	Alcohol(%)= 145.5 - 13.81 X_1 +3.06 X_2 - 0.0389 X_3 + 0.3333 X_1^2 - 0.444 X_2^2 + 0.002222 $X_1.X_3$ - 0.00444 $X_2.X_3$	0.9924
TSS(ºBx)	TSS(ºBx)=208.2- 20.17 X_1-1.06 X_2+ 0.0106 X_3+ 0.5000 X_1^2- 0.222 X_2^2+ 0.000022 X_3^3 + 0.00444 $X_2.X_3$	0.9934

X_1= Sugar (%); X_2= Yeast (g); X_3= Water (ml); R^2= Determination Coefficient

The effect of parameters on alcohol (%) and TSS (ºBx) were described in Table 3. Coefficient of determination (R^2) was used to evaluate the goodness of fitted model. The value $R^2 > 0.99$ implying that at least 99% of the variability in the response could be explained by the second-order model equations.

The larger the magnitude of T and smaller the value of P, the more significant is the corresponding coefficient term. From Table 4, the effects of all the linear and only X_1^2 (the interaction of sugar and sugar) were found to be highly significant ($P<0.05$) on the alcohol (%). A positive sign of the coefficient represents a synergistic effect, while a negative sign indicates an antagonistic effect. The effects of X_1(sugar) and X_1^2(the interaction of sugar and sugar) were found to be highly significant on TSS in Table 5.

Table 4: Estimated Regression Coefficients for Experimental Alcohol (%)

Term	Coef	SE Coef	T-value	P-value
Constant	7.000	0.129	54.22	0.000
X_1	1.8750	0.0791	23.72	0.000
X_2	0.3750	0.0791	4.74	0.005
X_3	0.2500	0.0791	3.16	0.025
X_1^2	0.750	0.116	6.45	0.001
X_2^2	-0.250	0.116	-2.15	0.084
X_3^2	0.000	0.116	0.00	1.000
X_1X_2	0.000	0.112	0.00	1.000
X_1X_3	0.250	0.112	2.24	0.076
X_2X_3	-0.250	0.112	-2.24	0.076

Table 5: Estimated Regression Coefficient for Experimental TSS (ºBx)

Term	Coef	SE Coef	T-value	P-value
Constant	7.000	0.129	54.22	0.000
X_1	2.0000	0.0791	25.30	0.000
X_2	-0.1250	0.0791	-1.58	0.175
X_3	0.1250	0.0791	1.58	0.175
X_1^2	1.125	0.116	9.67	0.000
X_2^2	-0.125	0.116	-1.07	0.332
X_3^2	-0.125	0.116	-1.07	0.332
X_1X_2	0.000	0.112	0.00	1.000
X_1X_3	-0.000	0.112	-0.00	1.000
X_2X_3	0.250	0.112	2.24	0.076

A contour plot is the projection of the response surface as a two-dimensional plane. This analysis gives a better understanding of the influence of variables and their interaction on the response. In Figure 1, the higher the sugar concentration, the higher the alcohol content was. This is because sugar had significant effect on alcohol content (P=0.000) with ANOVA analysis. At the highest yeast

concentration, the highest alcohol content could be seen. The alcohol content was found to be 9% when the sugar concentration was 23% (the highest). From this, it could be said that the alcohol content and sugar concentrations were directly related.

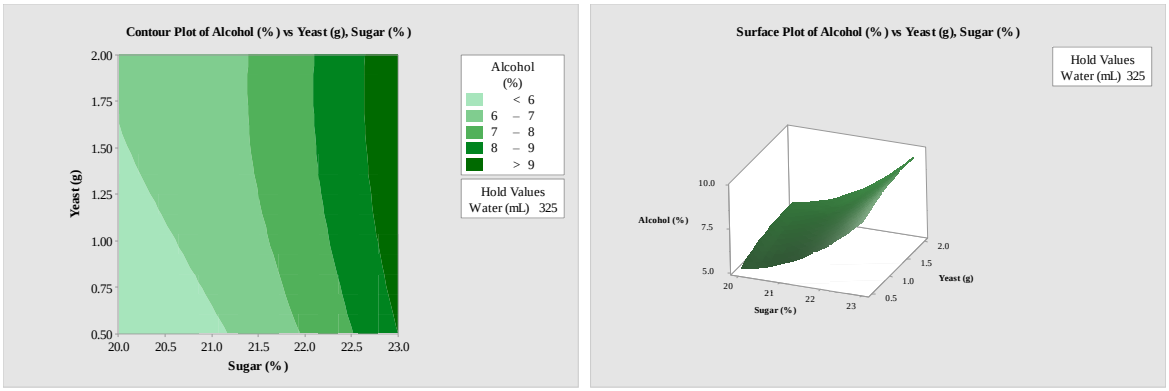


Figure 1: Surface and Contour Plots of the Interaction of Sugar-Yeast (Water 325mL) and Their Influence on Alcohol Content

In Figure 2, the highest alcohol could be obtained when sugar concentration was the highest. The alcohol content was directly proportional to the sugar concentration.

In Figure 3, it could be seen that as the yeast concentration was larger, the alcohol content was larger as the yeast concentration had P value of 0.05.

Figures 4, 5 and 6 represented the influences of sugar, water and yeast concentrations on total soluble solids.

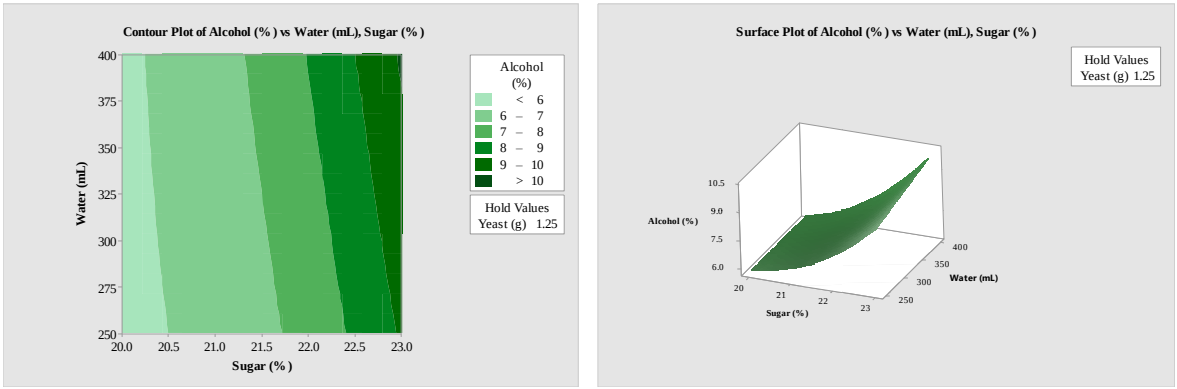


Figure 2: Surface and Contour Plots of the Interaction of Sugar-Water (Yeast=1.25g) and Their Influence on Alcohol Content

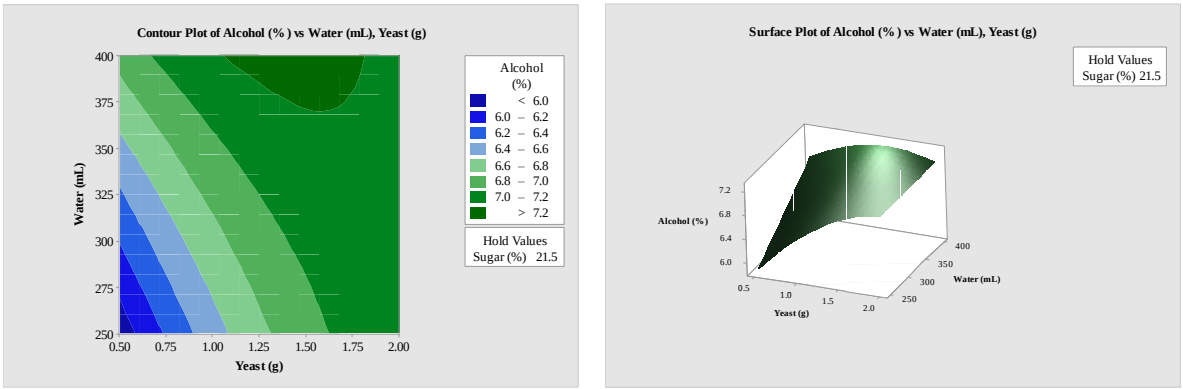


Figure 3: Surface and Contour Plots of the Interaction of Yeast-Water (Sugar=21.5%) and Their Influence on Alcohol Content

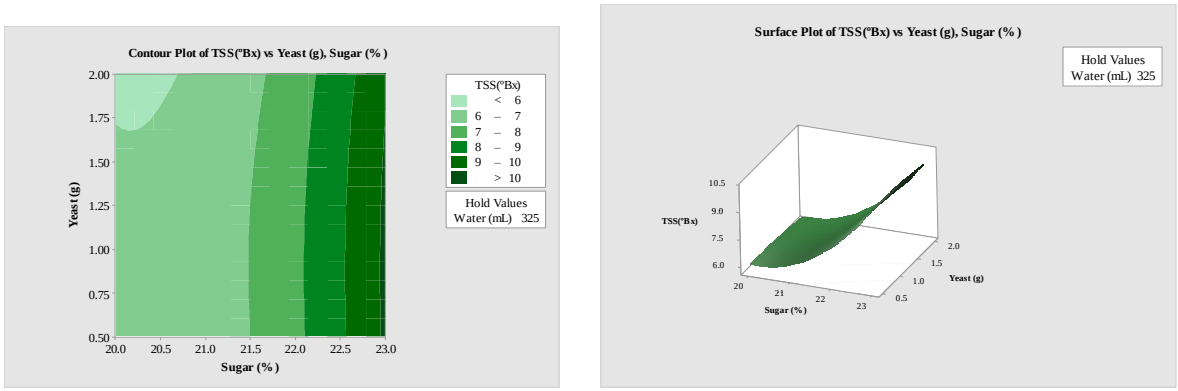


Figure 4: Surface and Contour Plots of the Interaction of Sugar-Yeast (Water=325ml) and Their Influence on Total Soluble Solids (TSS)

In Figure 4,when the sugar concentration was higher, the total soluble solids was higher and yeast had no effect on total soluble solids that is why sugar had P value of 0.000 and yeast had P value of 0.175 (not significant).

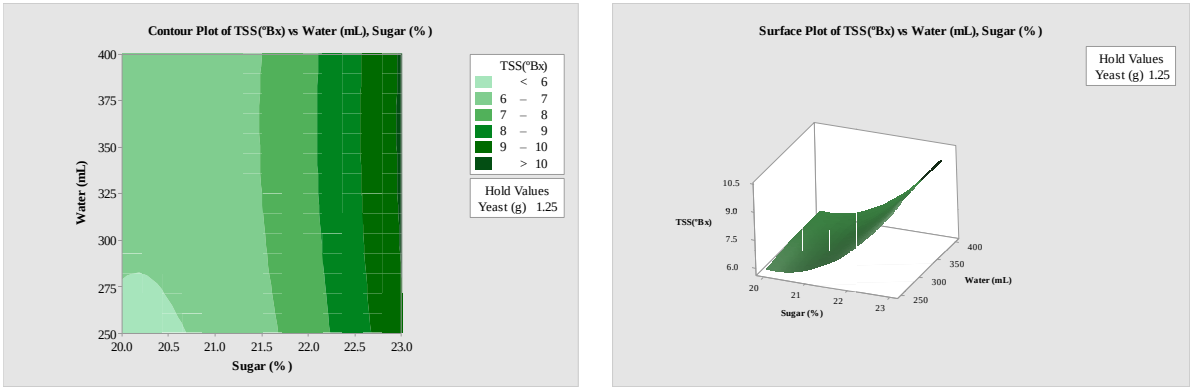


Figure: 5 Surface and Contour Plots of the Interaction of Sugar-Water (Yeast=1.25g) and Their Influence on Total Soluble Solids (TSS)

In Figure 5, whatever the water concentration, the total soluble solids content was high when the sugar concentration was high. Sugar only had significant effect and water had no significant effect with P value of 0.175.

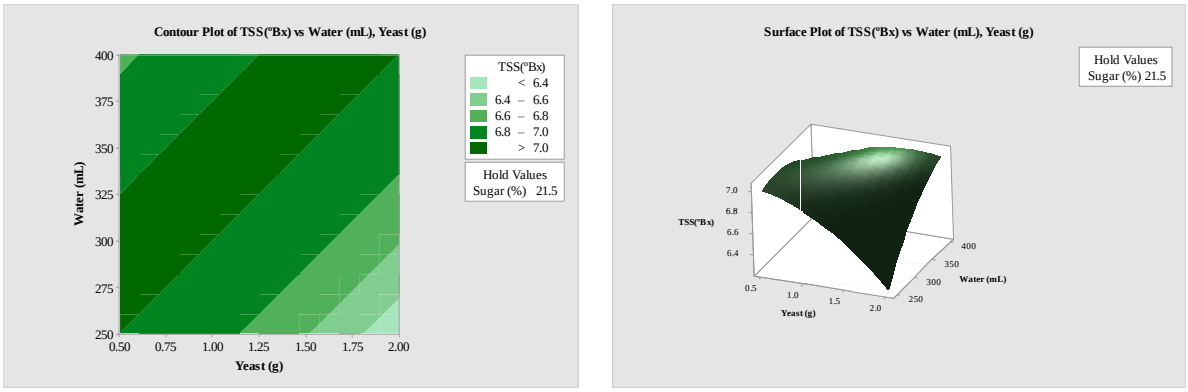


Figure: 6 Surface and Contour Plots of the Interaction of Yeast-Water (Sugar=21.5%) and Their Influence on Total Soluble Solids (TSS)

In Figure 6, there was no effect of water concentration on total soluble solids but the yeast concentration had effect on total soluble solids. The total soluble solids content was the highest at the lowest yeast concentration. The higher the yeast concentration was, the lower the total soluble solids content.

The proposed optimum condition of the wine were prepared and the physico-chemical characteristics of mango wine are described in Table 6. The pH value was 3.91 and it was within the pH range of 3.0-4.0 (Philippine FDA standard for fruit wine). The result of alcohol was 7.4% and total soluble solids(TSS) was 7.0 ^{o}Bx. The phytochemicals screening test of mango wine was done and represented in Table 7.

Table 6: Physico-chemical characteristics of mango wine product

No.	Test parameter	Result
1.	pH	3.91
2.	Alcohol (%)	7.4
3.	Total Soluble Solids (^{o}Bx)	7.0

Table 7: Phytochemicals screening test

No.	Type of compound	Extract	Reagent used	Observation	Results
1.	Alkaloid	1% HCl	Mayer's reagent	Cream colour (turbid)	+
			Wagner's reagent	Reddish brown ppt	
			Dragendorff's reagent	Orange ppt	
			Hager's reagent	Yellow ppt	
2.	Carbohydrate	H_2O	10% α-naphthol & conc. H_2SO_4	Red ring	+
3.	Glycoside	H_2O	10% lead acetate solution	White ppt	+
4.	Phenol	H_2O	5% $FeCl_3$ solution	Brownish green colour	+
5.	α- amino acid	H_2O	Ninhydrin reagent	Light purple colour	+
6.	Saponin	H_2O	H_2O	No persistent foam	-
7.	Tannin	H_2O	1% Gelatin & 10% NaCl solution	No ppt	-
8.	Flavonoid	70% EtOH	Mg ribbon & conc. HCl	Pink colour	+
9.	Steroid	Petroleum ether	Acetic anhydride & conc. H_2SO_4	Bluish green	t
10.	Terpenoid	Petroleum ether	Acetic anhydride & conc. H_2SO_4	Pink	t
11.	Reducing sugar	H_2O	Fehling's solution	Brick red ppt	+
12.	Starch	H_2O	Iodine solution	Purple blue	+
13.	Cyanogenic glycoside	Powder	H_2O , conc. H_2SO_4 sodium picrate paper	No colour change	-

* DCPT lab (+) presence, (-) absent, Et OH = ethanol, ppt = precipitate, t = trace

From Table 7, alkaloid, carbohydrate, glycoside, phenol, α- amino acid, flavonoid, reducing sugar and starch were found in the mango wine. Trace amounts of steroid and terpenoid were examined. Saponin, tannin and cyanogenic glycoside were absolutely absent.

Among the possible health effects of phytochemicals, the antioxidant activity was examined with DPPH radical scavenging assay. Different concentrations (6.25, 12.5, 25, 50, 100 and 200 ppm of mango wine and standard gallic acid were measured at 517nm. Figure 7 showed the result of percent inhibition.

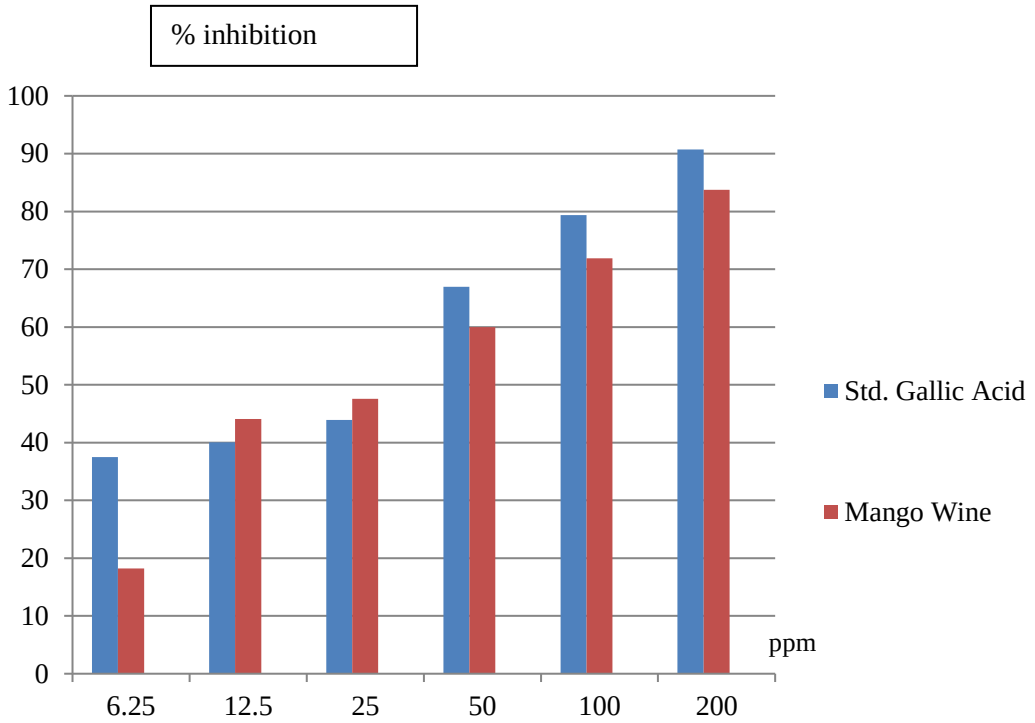


Figure 7: The percent inhibition of different concentration of Mango wine and Standard Gallic acid

From Figure 7, percent inhibition of mango wine and standard gallic acid at different concentrations could be seen. The percent inhibition of mango wine was lower than gallic acid for most of the concentration. Since mango wine showed inhibition effect, it could be said that mango wine had substantial antioxidant activity comparing with standard gallic acid.

Table 8: Free radical scavenging (DPPH) and IC_{50} of standard gallic acid and mango wine

Sample	Free radical scavenging (DPPH) % (ppm)						IC50 (ppm)
	6.25	12.5	25	50	100	200	
Std Gallic acid	37.5	40.02	43.9	66.96	79.36	90.7	31.61
Mango Wine	18.22	44.09	47.58	59.99	71.9	83.72	29.86

* DCPT lab

IC_{50} (EC_{50}) is the concentration of the sample leading to 50% reduction of initial DPPH concentration. It was obtained from the linear regression of plots of mean % of the antioxidant activity against the concentration of the test sample obtained from replicate assays. A compound acts as a powerful antioxidant if the value of the IC_{50} is less than 50, strong if the IC_{50} value is 50-100, moderate if the IC_{50} value is 100-150, and weak if the IC_{50} appreciating 150-200.

Table 8 showed the IC_{50} of standard gallic acid and mango wine. The results from Table 4 indicated that the mango wine had a very strong antioxidant activity. Percentage inhibition above 70% started from a concentration of 100 ppm. The mango wine showed a very strong antioxidant activity with IC_{50}<50.

4 CONCLUSION

A Box-Behnken, second order polynomial regression model design was proven to be a suitable response surface methodology to determine the effects of operative variables in mango wine preparation as the analysis of variance showed a high coefficient of determination (R^2> 99%). Studies from this design, the optimum conditions for mango wine fermentation were water and mango pulp ratio (2:1), yeast concentration 2.5 g/L and initial sugar concentration 22%. In this fermentation process, the mean

values obtained were alcohol content 7.4%, pH 3.91 and total soluble solids 7 (ºBx). It is suggested that Box-Behnken design is useful to provide light wine with desired quality.

Majority of the health-promoting phytochemicals are present in mango wine. Due to the presence of phenolic compounds and from the antioxidant assays, it was showed that mango wines possess a strong antioxidant capacity (IC_{50}<50). This study provides an avenue and scope to ferment mango fruits into value-added products such as wine which can be used as functional beverage that provides possible health effects to the consumers round the year.

5 ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Department of Chemical Engineering, YTU for supporting facilities required for this experiment. The authors express their sincere gratitude to Department of Chemical Engineering, YTU and DCPT lab for providing facilities required for this experiment.

REFERENCES

- Ana Ljevar, Natka Ćurko*, Marina Tomašević, Kristina Radošević, Višnja Gaurina Srček and Karin Kovačević Ganić. (2015). Phenolic Composition, Antioxidant Capacity and *in vitro* Cytotoxicity Assessment of Fruit Wines, University of Zagreb, Faculty of Food Technology and Biotechnology, Pierott ij eva 6,HR-10000 Zagreb, Croatia.
- Jie, Chan Li. (2014).Effects of Sugar Concentration and Yeast Inoculation Strategy on Mango Wine Fermentation .MSc. Thesis, National University of Singapore.
- Kumar,A .(2009) *Phytochemicals Investigation on a Tropical Plant, Syzygiumcumini from Kattuppala yam, Erode District, Tamit Nadu, South India*, PakistonJournal of Nutrition 8 (1), pp83-85,2009.
- Medina, J., & García, H. (2002). Mango: Post-harvest operations. *Mejia, D. and Lewis, B.(eds)*.
- Myat, K., Canavari, M., & Darnhofer, I. (2013). *Export Conditions of Myanmar mango: Hindrances and Opportunities in the Supply Chain*: LAP LAMBERT Academic Publishing.
- Miljić, U. D., & Puškaš, V. S. (2014). Influence of fermentation conditions on production of plum (Prunus domestica L.) wine: A response surface methodology approach. *Hemijska industrija, 68*(2), 199-206.

Investigation of Phytochemicals, Antioxidants and Antimicrobial Activities in Sacha Inchi (*Plukenetia volubilis*) Press Cake of Myanmar Origin

Su Lwin Htike*, Si Si Win , Win Nandar Thet

Department of Chemical Engineering, Yangon Technological University, Myanmar
*Corresponding Author (sulwinhtike.honey19@gmail.com)

ABSTRACT

Bioactive substances in Sacha inchi (*Plukenetia volubilis*) press cake (powder, methanol, ethanol) extracts were investigated against with six clinically important organisms, namely *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albican and E. coli* performed by Agar Plate Diffusion Method. The antimicrobial study showed that the extracts of press cake sample can inhibit the tested organisms to some extent. From the screening tests, phytochemicals such as alkaloids, glycosides, phenolic compounds, saponins and amino acids contain in the press cake sample, except starch and cyanogenic glycosides which were totally absent in it. The content of total phenolics extracted from press cake was found as 15.4 μ mol of gallic acid equivalents per gram of dried press cake. Determination of total antioxidant activity was carried out by the assay method of DPPH and the resulting data, 8.51 mgAA was observed. These experimental data demonstrates that Sacha inchi press cake present in Myanmar is one of the potential plant for therapeutic use and provide health benefits for human.

KEYWORDS: Antimicrobial, Agar Plate Diffusion Method, Cyanogenic Glycoside, DPPH, Gallic Acid Equivalent

1 INTRODUCTION

Sacha inchi (*Plukenetia volubilis* Linneo), also known as "Inca peanut" or "mountain peanut", is a plant (herbaceous perennial), belonging to the Euphorbiaceae family that is native to the Peruvian tropical jungles of South America , at altitudes between 200 and 1500m [8]. The plant produces star-shaped green fruits, which yield edible dark brown seeds, slightly enlarged in the center and squashed towards the edges (Fanali *et al*., 2016). The nut (seeds) can be considered as an excellent source of 27 – 30 % protein (including essential amino acids such as cysteine, tyrosine, threonine, and tryptophan) and 40 – 60 % oil. The oil comprises approximately 90% unsaturated fatty acids (oleic ω - 9, linoleic ω - 6, linolenic ω - 3) and is rich in antioxidants (tocopherols and beta-carotene) (Fu *et al.,* 2014; Pereira de Souza *et al*., 2013) and contains heat labile substances with a bitter taste (Krivankova *et al*., 2012).

Sacha inchi (SI) seeds are used in different forms by the Amazonian population. The oil is used in the preparation of various meals, the seeds are consumed roasted and the leaves are cooked and consumed. SI seeds are also used as the traditional remedy in the Amazon region to treat rheumatic problems and aching muscles [9]. The oil has slight characteristic odour as well as slight typical taste as beans. It has a caloric value of 576kcal/100g sample, calculated from the contents of fat, protein and carbohydrate (Gutiérrez *et al*., 2011). It has a great potential for applications in the food and pharmaceutical industries (Nascimento *et al.,* 2013).

Besides being a source of nutrient, foods particularly plant food are a rich source of bioactive phytochemicals or bionutrients (Kumar *et al*., 2009). Phytochemicals in fruits, vegetables, grains, and other plant foods have been linked to reductions in the risk of major chronic diseases It is estimated that more than 5000 phytochemicals have been identified but a large percentage still remain unknown and need to be identified before their health benefits are fully understood (Liu, 2003).

Recently, many diseases such as cancer, diabetes, arteriosclerosis, inflammatory disease, autoimmunity, cardiovascular disease, and Alzheimer's have been associated with the increase of reactive oxygen species (ROS) or the inability of the organism to reduce these ROS that were normally produced by the organism cells, a process known as oxidative stress. Antioxidants are

important substances that have the ability to protect the organism from the damage caused by the oxidative stress. Due to this ability, there is a special interest in the presence of natural antioxidants in medicinal plant that may help an organism to keep the normal balance of ROS (Nascimento *et al.*, 2013).

Figure 1: Sacha inchi plant and seeds

In Myanmar, SI recently has potential as the new economic crop and the healthy oil seed. However, the information of SI in Myanmar is still limited because it has been newly introduced oilseed crop. It has been imported for plantation throughout the country (Kachin State, Kayin State, Shan State, Mon State, Magwe Division, Ayeyarwaddy Division, Yangon Division, Bago Division). The composition of Sacha inchi can be different from its native country due to its different varieties and geographical conditions.

Vegetable oil processing releases a large quality of pressed cake which still remain high nutritional compound. However, these by-products are usually used as feed meals or as fertilizers. Utilization of the by-products of sacha inchi oil could be beneficial in value adding as well as reducing the waste. The sieved ground pressed cake can be used as a source of protein in bakery products (Thanatpant Tanasamrit *et al.,* 2016).

Sacha inchi should be known and need in searching for health effect what compounds are present in it. Hence, keeping this view in mind, the present study has been made to investigate the phytochemical constituents and antioxidant activity of the Sacha inchi press cake as well as its possible actions like antimicrobial activity, in order to find some new industrial applications for this emerging new crop.

2 MATERIALS AND METHODS

2.1 Collection and Identification of Plant Materials

The plant, flowers and seeds of the sample supported from Unicon Sacha inchi Factory were identified and authenticated with the help of a botanist from University of Yangon.

2.2 Sample Preparation

The press cakes were dried first to reduce moisture, reduce particle size and packed in in double PE bags, within an airtight container and stored at room temperature until the beginning of the experiments.

2.3 Proximate Compositional Analysis of Press Cake

Analysis of proximate composition content (moisture, ash, protein, fat and carbohydrate (by difference)) were performed based on AOAC methods (2000) and fiber by Fiber Cup Method.

2.4 Preparation of Extracts

The aqueous extract of press cake was prepared by soaking 100g of dried powdered samples in 200 ml of distilled water for 12 hr. The extracts were filtered using Whatman filter No 1.

2.5 Preliminary Phytochemical Screening

Sample extracts (aqueous, ethanol and methanol as soaking solvents) were prepared for screening procedure.

2.6 Antioxidant Activity

DPPH radical scavenging activity was determined in this experiment according to the method of Zhao et al., (2011) with modification. DPPH reagent was prepared by dissolved 0.1 mM DPPH in 95% methanol. Approximately 0.5 ml of sample solution was added with 2 ml of DPPH reagent. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at the absorbance at 517nm. The result was expressed as mgAA.

2.7 Total Phenolic Content

Total phenolic content of the extract was determined by the Folin-Ciocalteu method. 200 ml of the crude extract (1 mg/ml) were made up to 3 ml with distilled water, mixed thoroughly with 0.5 ml of Folin-Ciocalteu reagent for 3 min, followed by the addition of 2 ml of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650nm. The result was expressed as mg gallic acid equivalent (GAE) per gram of dried press cake.

2.8 Antimicrobial Studies

The six clinically important microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albican , E. coli* were used to test against with Sacha inchi press cake powder for antimicrobial studies by Agar Plate Diffusion Method.

3 RESULTS AND DISCUSSIONS

According to features of the plant, Sacha inchi present in Myanmar came from the scientific name of the plant *Placentia volubilis* and contains in Euphorbiaceae Family. The detailed description of the plant is shown in (Tables 1, 2).

Table 1: Scientific Classification*

Scientific classification	*Plukenetia volubilis*
Myanmar name	Kyae - pe
English name	Sacha inchi, Sacha peanut
Family	Euphorbiaceae

*by botanist, Yangon University

Table 2: Description of Sacha inchi Plant Identification

Habit		Perennial plant, climber
Leaves		Alternate, simple, petiolate, exstipulate, hairy leaf, heart shaped, serrated
Inflorescences		Axillary, cymose
Fruits		Capsule, green and ripen blackish brown
seeds		Endospermic with a straight embryo, oval, dark brown seeds are covered with a whitish film
Male flower	Flower	Small, bracteates, pedicellate, incomplete, unisexual, regular, actinomorphic, 4 – 6 merous cyclic
	Calyx	Sepals 4, aposepalous, valvate, sepaloid
	Corolla	absent
	Androecium	Stamens infinity, apostemanous, fliament unequal, anther dithecous, extrorse, exerted, adnate fixtion, longitudinal dehiscences
Female flower	Flower	Bracteates, bracteolate, pedicellate, incomplete, unisexual, regular, actinomorphic, 4 – 6 merous, cyclic, hypogynous
	Calyx	Sepals, 4 - 6, aposepalous, valvate, sepaloid, persistant, inferior
	Corolla	absent
	Gynoecium	Carpels (4 - 6), tetra or hexa carpellary, syncarpous, tetra or hexa locular, one ovule in each locule, axile placentation, stigma tetra or hexa fid, style long, superior

The press cake has moisture content of 7.52% and 6.21% of ash. But it has little fat 4.63% as these are the by-products of oil extraction and is rich in protein (56%) containing essential amino acids that are vital macronutrient and essential for sustenance of life.

Table 3: Proximate Composition of Press Cake Sample*

Sr. No	Test Parameter	Test Method	Result
1.	Moisture (g/100g)	AOAC- 2000 (930.04)	7.52%
2.	Ash (% dry basis)	AOAC- 2000 (930.05)	6.21%
3.	Crude Protein (N x 6.25) (% dry basis)	AOAC- 2000 (920.152) (Kjeldahl Method)	56.00%
4.	Crude Fiber	AOAC- 2000 (978.10) Fiber Cap Method	1.52%
5.	Crude Fat (ether extract)	AOAC (Buchi Soxhlet Method)	4.63%
6.	Carbohydrate	By difference	24.12%
7.	Energy value (calculated) (kcal/ 100g)		365

* FIDSL lab

From the results obtained in the present investigation phytochemicals (Table 4), the extracts sample of press cake showed the absence of flavonoids, cyanogenic glycosides, tannins and starch, but steroids, terpenoids as well as reducing sugars are found in trace amount. Alkaloids, carbohydrates, glycosides, amino acids, phenolic compounds and saponins are present in it. The presence of phytochemicals in the press cake indicates that it can also have various medicinal values such as anti-inflammatory, anti-diabetic, analgesic activities and also have central nervous system.

Plants rich in secondary metabolites, including phenolics, flavonoids and carotenoids have antioxidant activity due to their redox properties and chemical structures. The methanolic extract of SI press cake had antioxidant activity against the free radicals investigated. The DPPH radical is widely used in assessing free radical scavenging activity because of the ease of the reaction. DPPH scavenging activity was 8.51 mgAA. The total phenolic content of SI press cake was determined using Folin-

Ciocalteu reagent. The results showed that SI press cake was 15.4 μ mol of gallic acid equivalents per gram of dried press cake described in (Table 5).

Much of the protective effect of plants (fruits and vegetables) has been attributed by phytochemicals, which are the non-nutrient plant compounds. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. Thus, the present work had been undertaken to examine phytochemicals in Sacha inchi nut and its various parts.

From the antimicrobial studies (Table 6), the press cake powder sample (not extract) tested against with six clinically important microorganisms can inhibit only upon *Pseudoonas* species while its methanol and ethanol extracts can inhibit all tested organisms, but ethanol extracts have more inhibitory activity than methanol extracts except on *Pseudomonas* species. According to the experimental datas, ethanol extracts have strong inhibitory power upon *B. subtilis* and *E. coli*. The antimicrobial activity of the press cake is due to the presence of polyphenols. These all indicates the medicinal value and supports the claim of the traditional healers.

Table 4: Phytochemical Screening Test*

No	Type of compound	Extract	Reagent used	Observation	A
1.	Alkaloids	1% HCl	Mayer's reagent	White ppt	+
			Wagner's reagent	Brown ppt	
			Dragendorff's reagent	Orange ppt	
			Hager's reagent	Yellow ppt	
2.	Carbohydrate	H_2O	10% - α naphthol & conc H_2SO_4	Red ring	+
3.	Glycosides	H_2O	10% lead acetate solution	White ppt	+
4.	Phenolic compounds	H_2O	5% $FeCl_3$ solution	White ppt	+
5.	α amino acid	H_2O	Ninhydrin reagent	Purple colour	+
6.	Saponin	H_2O	H_2O	Persistant foam	+
7.	Tannin	H_2O	1% lead acetate	No ppt	-
8.	Flavonoids	70% EtOH	Mg ribbon & conc HCl	Pink colour(+) / No pink colour(-)	-
9.	Steroid	Petroleum ether	Acetic anhydride & Conc H_2SO_4	Bluish green	t
10.	Terpenoids	EtOH	$CHCl_3$ & Conc H_2SO_4	Reddish brown	t
11.	Reducing sugar	H_2O	Fehling's solution	Yellow ppt	t
12.	Starch	H_2O	Iodine solution	Brown ppt	-
13.	Cyanogenic glycoside	Powder	H_2O , Conc H_2SO_4 sodium picrate paper	No colour change	-

* DCPT lab (+) presence, (-) absent, Et OH = ethanol, ppt = precipitate,
t = trace A = Press Cake

Table 5: Antioxidant and Total Phenolic Content

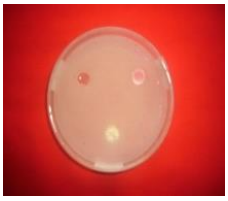
Proterties	Values
Antioxidant activity DPPH value (mgAA)	8.51 mgAA
Total phenolic content (μmol)	15.4 μ mol

AA – Ascorbic acid

Table 6: Antimicrobial Activity*

Diameter of inhibitory zone							
Sample	Solvent	*B – sub*	*S – aureus*	*Pseudomons*	*B – pumilus*	*Candida*	*E. coli*
Press Cake	Powder	-	-	11 mm (+)	-	-	-
	Et OH	15 mm (++)	13 mm (+)	11 mm (+)	13 mm (+)	13 mm (+)	15 mm (++)
	Me OH	12 mm (+)	11 mm (+)	12 mm (+)	12 mm (+)	12 mm (+)	13 mm (+)
	control	-	-	-	-	-	-

*DCPT lab, (-) no zone was observed. Agar well (Disc diameter) – 10 mm
10 mm ~ 14 mm (+); 15 mm ~ 19 mm (++); 20 mm and above (+++).

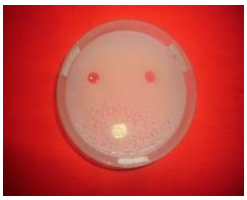


Bacillus subtilis

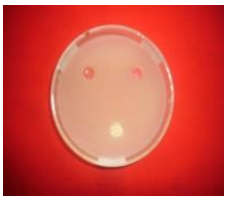
Staphylococcus aureus

Pseudomonas aeruginosa

Bacillus pumilus



Candida albican



Escherichia coli

However, there is a need for more information about the health and possible risks to ensure the efficiency and safety of Sacha inchi used as dietary supplement. The present work suggests to improve nutrition and health of consumers by getting antioxidants from the Sacha inchi, but much more strong scientific evidence is required in further studies.

4 CONCLUSION

In the present study, it was found that some of the biologically active phytochemicals were present in the extracts of sacha inchi press cake. Medicinal properties (viewed from experimental studies) of the extracts may be due to the presence of above mentioned phytochemicals. Even though, this is only a preliminary study of the occurrence of certain properties of the tested samples, an in-depth study will provide a good concrete base of all the phytochemicals functions above.

Further studies should be carried out to study the value added products from Sacha inchi (kyae pe). This work needs to be corroborated in humans using quantities that are relevant to feasible levels of intake for foodstuffs i.e required to affect benefits.

5 ACKNOWLEDGEMENTS

The authors would like to express our gratitude to the Department of Chemical Engineering, Yangon Technological University and Unicon Sacha inchi production factory for providing all the necessary supports.

REFERENCES

- Fanali, C., Dugo, L., Cacciola, F., Beccaria, M., Grasso, S., Dacha, M., ... & Mondello, L. (2011). Chemical characterization of Sacha Inchi (Plukenetia volubilis L.) oil. *Journal of agricultural and food chemistry*, *59*(24), 13043-13049.
- Fu, Q., Niu, L., Zhang, Q., Pan, B. Z., He, H., & Xu, Z. F. (2014). Benzyladenine treatment promotes floral feminization and fruiting in a promising oilseed crop Plukenetia volubilis. *Industrial Crops and Products*, *59*, 295-298.
- Gutiérrez, L. F., Rosada, L. M., & Jiménez, Á. (2011). Chemical composition of sacha inchi (Plukenetia volubilis L.) seeds and characteristics of their lipid fraction. *Grasas y aceites*, *62*(1), 76-83.
- Krivankova, B., Cepkova, P. H., Ocelak, M., Juton, G., Bechyne, M., & Lojka, B. (2012). Preliminary study of diversity of Plukenetia volubilis Based on the morphological and genetic characteristics.
- Kumar, A., Ilavarasan, R., Jayachandran, T., Decaraman, M., Aravindhan, P., Padmanabhan, N., & Krishnan, M. R. V. (2009). Phytochemicals investigation on a tropical plant, Syzygium cumini from Kattuppalayam, Erode district, Tamil Nadu, South India. *Pakistan Journal of Nutrition*, *8*(1), 83-85.
- Liu, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American journal of clinical nutrition*, *78*(3), 517S-520S.
- Nascimento, A. K. L., Melo-Silveira, R. F., Dantas-Santos, N., Fernandes, J. M., Zucolotto, S. M., Rocha, H. A. O., & Scortecci, K. C. (2013). Antioxidant and antiproliferative activities of leaf extracts from Plukenetia volubilis Linneo (Euphorbiaceae). *Evidence-Based Complementary and Alternative Medicine*, *2013*.
- Pereira de Souza, A. H., Kirie Gohara, A., Cláudia Rodrigues, Â., Evelázio de Souza, N., Vergílio Visentainer, J., & Matsushita, M. (2013). Sacha inchi as potential source of essential fatty acids and tocopherols: multivariate study of nut and shell. *Acta Scientiarum. Technology*, *35*(4).
- Q.Liu et al. (2014). "Chemical Composition and Oxidative Evolution of Sacha Inchi (*Plukenetia volubilis* L.) oil from Xishuangbanna (China) ", *Grasas y acettes*, *65(1)*, 1-9.
- Souza, A . H . P . et al. (2013). " Sacha inchi as Potential Source of Essential Fatty Acids and Tocopherols: Multivarate Study of Nut and Shell", *Acta Scientiaraum Technology, Maringa, 35*, 757-763.
- Thanatpant Tanasamrit et al. (2016). "Extraction and Determination of Chemical and Functional Properties of Protein from Sacha inchi (*Plukenetia volubilus* L.) Press Cake", International Science on Food and Applied Bioscience,79-89.

Optimization of Lactic Acid Production from Fermentation of Vegetable Wastes through Response Surface Method

Shwe Zin Moe*, Si Si Win

Department of Chemical Engineering, Yangon Technological University, Yangon, Myanmar
*Corresponding Author (szmoe1504@gmail.com)

ABSTRACT

This study introduces to a process for the fermentative production of lactic acid from vegetable wastes (cabbage and cauliflower leaves wastes) by using indigenous microorganisms. Cabbage and cauliflower leaves are rich carbohydrate and nitrogen source vegetable so they are suitable as a cheaper source of raw material for the production of lactic acid. The wastes contained about (w/w) 90% moisture, 4% carbohydrate, 2% reducing sugar and 1% protein. Optimization, quantification and kinetic parameter were evaluated for lactic acid production during anaerobic digestion of vegetable waste using salts through Box-Behnken design (BBD) of Response Surface Method (RSM). RSM was used to investigate the effects of four fermentative variable parameters: waste to water ratio (1:0 to 1:2), salt concentration (1% to 5%), initial pH control (6 to 8) and fermentation Period (1day to 5days) on the concentration of lactic acid. Outcomes revealed a relationship between lactic acid concentration and waste to water ratio, salt concentration and fermentation period. RSM was found to be useful in optimizing and determining the interactions among process variables in lactic acid production by fermentation with salt.

KEYWORDS: Vegetable Wastes, Lactic Acid, Fermentation, Optimization, Response Surface Method.

1 INTRODUCTION

Lactic acid (LA) is GRAS (generally recognized as safe) grade one, being declared safe by the United States Food and Drug Administration. Lactic acid, one of the functional, valuable and versatile compounds has been utilized globally for synthesizing various compounds in food, textile, pharmaceutical, cosmetics and chemical industries and especially in the food industry. It is widely used in almost every segment of the food industry, where it serves in a wide range of functions, such as flavoring, pH regulation, and mineral fortification. It is also used for increasing the shelf life of meat and poultry products, pickled vegetables and beverages (Wee, Kim and Ryu, 2006). Lactic acid is also used in the pharmaceutical industry for preparation of electrolytes in many parenteral /intravenous solutions, tablets, prostheses, surgical sutures, and in controlled drug delivery systems (Lunt, 1998). Worldwide demand for lactic acid is growing at a rate of approximately 12-15% a year. Lactic acid production from agricultural crops such as wheat, corn and beet has recently received much attention because of the increasing demands for polylactic acid, which is used in biodegradable plastics (Akerberg and Zacchi, 2000). Biodegradable polylactic acid (PLA) that can be used to improve physical properties in the production of food packaging, plastic utensils, garbage bags and agricultural plastic sheeting, thereby replacing products made from petroleum. Lactic acid can be produced by fermentation process with microorganisms and chemical synthesis. The fermentation process has the advantage of being more cost effective and approximately 90% of all lactic acid worldwide is produced by bacterial fermentation (Zhou, Shanmugam, and Yomano, 2006).

Lactic acid can be produced biologically by submerged fermentation or solid state fermentation. Solid state fermentation is more advantageous than the submerged fermentation as it results in higher yields with more concentrated organic acids. But solid state fermentation occurs in the absence or near absence of free water. Low moisture content may lead to poor accessibility of nutrients resulting in poor microbial growth. Semi-solid fermentation provides high water activity to the solid substrate and also

provides carbohydrates, mineral nutrients, and nitrogen sources which make whole process more economical.

The parts of vegetables not used are discarded as wastes. Vegetable wastes are form part of organic wastes which are major source of foul smell originating from large municipal dumping sites and landfills. It has been reported that such wastes can be utilized in various ways so to reduce the load of organic matter in landfills and dumping sites. Anaerobic digestion provides proficient recovery of resource and its impact on environmental is lesser therefore it is evaluated favorably with alternative waste management processes, such as incineration, landfill and composting. Production of organic acids has been tried using organic wastes materials like kitchen wastes previously. Utilization of wastes for production of useful production is a way to reduce load at dumping sites and it also provides a better solution for production of useful chemicals in a way which is easy and cheap compared to direct chemical processes. Vegetable wastes could be utilized cheaply as substrate for microorganisms producing intermediate high value organic acids like lactic acid (Sailaja and Mohit S. Mishra, 2015).

Enormous amounts of fruit and vegetable waste are generated daily in large cities worldwide, and the effective disposal of such highly biodegradable waste is a challenge. In Yangon, the total solid waste generated over 1,900 tons/day, market wastes are 15% of the total solid waste generated and vegetable wastes are about 60% of market waste (YCDC, 2016). Cabbage and Cauliflower leaves are one of the major waste and source of foul smell originating from large municipal dumping sites and landfills.

The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the interactions between the components. In order to overcome this problem, optimization based on the statistical design experiments will be carried out. Response surface methodology (RSM) is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical inferences. The first step in process optimization is screening of the important variables, with an aim of limiting them to three, four, or five at maximum. Following the initial screening, the next step is optimizing the levels of these variables by employing Box-Behnken Design of RSM. Box-Behnken is three-level fractional factorial arrangement, which allows the efficient estimation of the first- and second-order coefficients of the quadrating mathematical model. Several researchers have applied this technique for optimization of different parameters (Coelho, 2010; Amenaghawon, 2013).

The objective of the present study was to investigate the production of lactic acid from the carbohydrate rich vegetable waste (cabbage and cauliflower leaves) by indigenous microorganisms during fermentation and to optimize the production of lactic acid using Box-Behnken design of Response Surface Methodology. Experiments were carried out under a variety of operational conditions defined by four independent variables (ratio of slurry and water, % salts concentration, incubation period and initial pH) in semi-solid fermentation. The role of each variable, their interactions and the predicted production of lactic acid during fermentation were determined by applying Box-Behnken design of Response Surface Methodology.

2 MATERIALS AND METHODS

2.1 Vegetable Wastes Collection, Preparation and Vegetable Wastes Fermentation

Cabbage and Cauliflower leaves waste was collected from Thiri Mingalar Market, Yangon. The 100 g of washed freshly vegetable wastes were cut into small pieces and blended with pure water in the various ratio (0:1 to 2:1) (v/w) using an electrical food processor to produce thick slurry. The pH of the thick slurry was adjusted using CaO for various initial pH (6 to 8) and NaCl also was added to waste slurry (1% to 5%). The prepared slurry was poured in 300 ml flasks and the flasks were closed tightly with rubber caps to achieve and maintain microaerophilic fermentation conditions. The spontaneous anaerobic submerged fermentation process was carried out by natural indigenous microorganisms at 37°C in incubator for 5 days. The fermented slurry was collected after every 24 hour to estimate the lactic acid concentrations.

2.2 Analytical Methods

Fermented medium was filtered through Whatman filter paper and the filtrate was used for estimation of pH, and lactic acid daily basis up to 3 days. Lactic acid production from vegetable wastes was determined by titrating the culture broth against 0.1 N NaOH using phenolphthalein as indicator. 25 ml of culture broth was taken from the fermented broth in a flask and boiled for 2 minutes to remove air. NaOH solution was added with continuous shaking till there was persistent pink color formation. Amount of lactic acid was determined as the quantity of NaOH utilized in titration multiplied by 90.08.

2.3 Experimental Design and Statistical Analysis

Response surface methodology (RSM) has been widely and successfully applied for evaluating the effect of process variables and optimization of various bioprocesses. Box-Behnken design (BBD) was employed to study the effect of four variable factors Ratio of water and waste slurry, Salt concentration, Initial pH and Incubation Period were studied on the effect of lactic acid production. Box-Behnken Design (BBD) of Response Surface Methodology (RSM) is chosen to analyze statistically and to develop the second order polynomial model for the effects of these three variables on the production of lactic acid. Box-Behnken design of RSM has three levels (low, medium, and high coded as -1, 0, +1) for variables , needs fewer experiments (27 runs), more efficient and easier to interpret (Bosque-Sendra and Pescarolo, 2001).

The number of experiments (n) required for the development of BBD is defined as $n = 2k (k - 1) + C_o$ where k is the number of experimental variables and C_o is the number of experiments repeated at the center point ($k = 4$; $C_o = 3$). As a result, a total of 27 runs of experiments to optimize ratio of water to waste slurry (0:1 to 2:1), Salt concentration (1% to 5 %), Initial pH (6 to 8) and Incubation Period (1 day to 5 days) for the production of lactic acid have to be performed. All other experimental conditions were kept constant during the experiments.

For statistical calculations, the three independent variables are designated as X_1, X_2, $X_3$and X_4 , respectively, and were coded according to the following equation.

$$x = (X-X_0)/\Delta X \quad (1)$$

Where, x is coded variable, X is natural variable, X_0 is the middle point (zero level) and ΔX is the step change that represents the difference between the successive levels. The range and levels of the variables investigated in this study were shown in Table 1.

Table 1: Process Variables for Lactic Acid Production by Box-Behnken Design

No.	Variable factors	Lower level (-1)	Middle level (0)	Upper level (+1)	Step change ΔX=difference between levels
1	Ratio of water to waste slurry (v:w), X_1	0:1	1:1	2:1	1
2	Salt concentration (%), X_2	1	3	5	2
3	Initial pH, X_3	6	7	8	1
4	Incubation period (day), X_4	1	3	5	1

3 RESULTS AND DICUSSIONS

3.1 Proximate Analysis of Vegetable Wastes (Cabbage and Cauliflower Wastes)

Proximate analysis results clearly depicts that cabbage and cauliflower waste contained about 90% moisture, 4% carbohydrate, 2% reducing sugar and 1% protein. Cabbage and cauliflower leaves are rich carbohydrate and nitrogen source vegetable so they are suitable as a cheaper source of raw material as compared to others for the commercial production of lactic acid.

3.2 Effect of the Water to Waste Slurry Ratio, Salt Concentration and Incubation Period on Lactic Acid Production

RSM was employed to check the best operating parameters and select optimum fermentative conditions. The average lactic acid concentration varied from 3.2 to 12.15g/l. The maximum and minimum scores were obtained in experiment no.1 and 18, respectively (Table 2). In experiment no.1, the level of water to waste slurry ratio, salt concentration, initial pH and Incubation Period were 0:1, 1%, pH 7 and 3 days, respectively while the experiment no.18 comprised 2:1 ratio of water to waste slurry, 3% salt concentration, pH 7 and 1 days Incubation Period, respectively.

Table 2: Experimental Design of Process Variables and Values of Experimental Data for Lactic Acid Production by Box-Behnken Design

No.	Ratio of water to waste slurry, X_1	Salt concentration	Initial pH, X_3	Incubation period (day), X_4	Lactic acid (g/l)	
					Experimenta	Predicted
1	0	1	7	3	12.15±0.30	11.75
2	2	1	7	3	4.00 ± 0.05	5.24
3	0	5	7	3	11.61 ± 0.10	11.05
4	2	5	7	3	5.13 ± 0.00	5.22
5	1	3	6	1	5.40 ± 0.00	4.76
6	1	3	6	5	5.81 ± 0.05	5.29
7	1	3	8	1	4.50 ± 0.00	4.71
8	1	3	8	5	5.58 ± 0.00	5.91
9	0	3	6	3	9.81 ± 0.00	10.74
10	2	3	6	3	4.50 ± 0.10	4.79
11	0	3	8	3	11.07 ± 0.10	11.25
12	2	3	8	3	5.31 ± 0.10	4.85
13	1	1	7	1	5.00 ± 0.05	5.34
14	1	5	7	1	5.09 ± 0.05	4.94
15	1	1	7	5	5.54 ± 0.05	6.16
16	1	5	7	5	5.72 ± 0.15	5.84
17	0	3	7	1	9.27 ± 0.10	9.39
18	2	3	7	1	3.20 ± 0.05	3.31
19	0	3	7	5	10.62 ± 0.20	10.34
20	2	3	7	5	4.37 ± 0.05	4.08
21	1	1	6	3	7.38 ± 0.10	7.02
22	1	5	6	3	5.81 ± 0.15	6.10
23	1	1	8	3	7.20 ± 0.00	6.74
24	1	5	8	3	6.75 ± 0.00	6.95
25	1	3	7	3	7.11 ± 0.00	7.08
26	1	3	7	3	7.07 ± 0.05	7.08
27	1	3	7	3	7.07 ± 0.05	7.08

In order to determine the maximum lactic acid concentration corresponding to the optimum levels of different parameters, a second order polynomial model was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second order polynomial model (Equation 2) explained the role of each variable and their second order interactions in producing lactic acid. The data fitted the following linear model:

$$\text{Lactic acid (g/l)} = -8.4 - 4.93\,X_1 - 1.20\,X_2 + 1.91X_4 + 5.07\,X_3 + 1.223\,X_1{}^*\,X_1 + 0.0033\,X_2{}^*X_2 - 0.3806\,X_4{}^*\,X_4 - 0.392\,X_3{}^*\,X_3 + 0.084\,X_1{}^*\,X_2 - 0.023\,X_1{}^*\,X_4 - 0.113\,X_1{}^*X_3 + 0.0056\,X_2{}^*X_4 + 0.141\,X_2{}^*X_3 + 0.084\,X_4{}^*X_3 \quad (2)$$

Where: X_1 is ratio of water to waste slurry, X_2 is Salt concentration (%), X_3 is pH and X_4 is Incubation period (days).

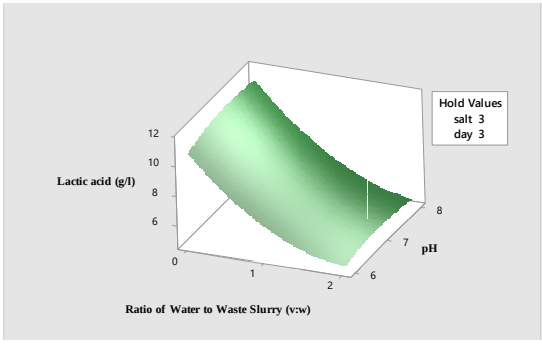
Coefficient of determination (R^2) was used to evaluate the goodness of fitted model. The value R^2> 0.97 implying that at least 97.54% of the variability in the response could be explained by the second-order model equations. The larger the magnitude of T and smaller the value of P, the more significant is the corresponding coefficient term.

The probability (p) values were used as a tool to check the significance of each of the coefficients. The smaller the value of p, the more significant is the corresponding coefficient term. In Table 3, the coefficient estimates of lactic acid production model showed that the level of ratio of water to waste slurry, salt concentration and Incubation period effect on lactic acid production but only the ratio of water to waste slurry were found to be highly significant (P<0.05) on the lactic acid production. A positive sign of the coefficient represents a synergistic effect, while a negative sign indicates an antagonistic effect. The interactions of the variables were shown in Figure 1(a-f) and 2(a-f).

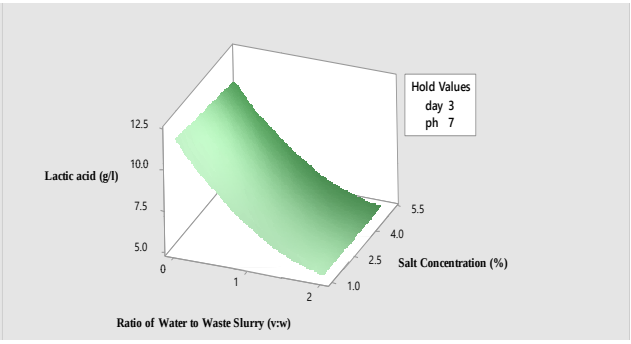
Table 3: Estimated Regression Coefficients and Corresponding P-values for The Production of Lactic cid

Term	Coefficient	SE Coefficient	p-Value
Constant	7.080	0.323	0.000
X_1	-3.086	0.162	0.000
X_2	-0.180	0.162	0.287
X_3	0.143	0.162	0.395
X_4	0.431	0.162	0.020
X_1* X_1	1.222	0.242	0.000
X_2* X_2	0.013	0.242	0.958
X_3* X_3	-0.392	0.242	0.132
X_4* X_4	-1.522	0.242	0.000
X_1* X_2	0.169	0.280	0.558
X_1*X_3	-0.113	0.280	0.695
X_1*X_4	-0.045	0.280	0.875
X_2*X_4	0.022	0.280	0.937
X_2*X_3	0.281	0.280	0.335
X_4*X_3	0.169	0.280	0.558

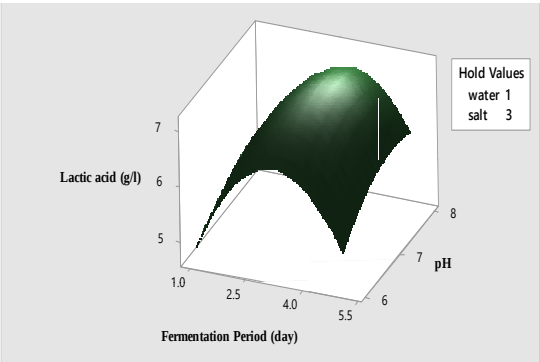
X_1 - Ratio of water to waste slurry, X_2 - Salt concentration (%), X_3 – pH, X_4 - Fermentation period (days)



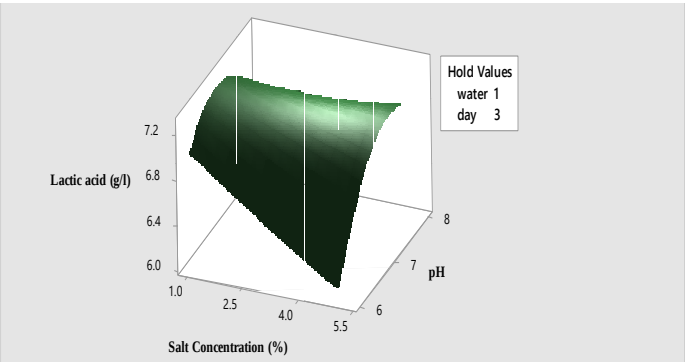
(a)



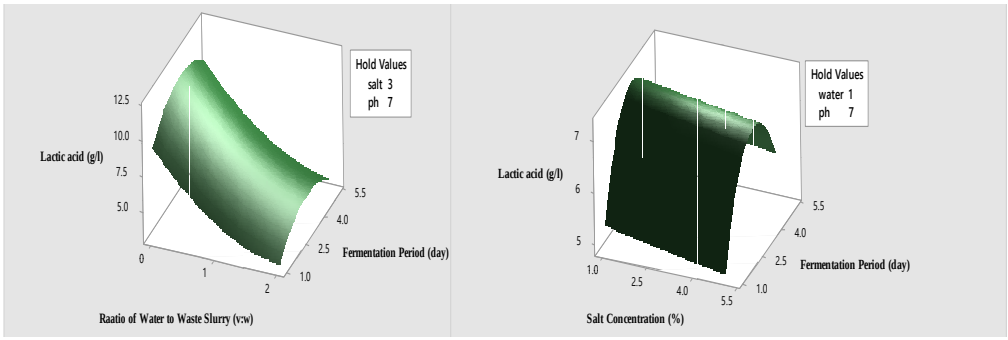
(b)



(c)

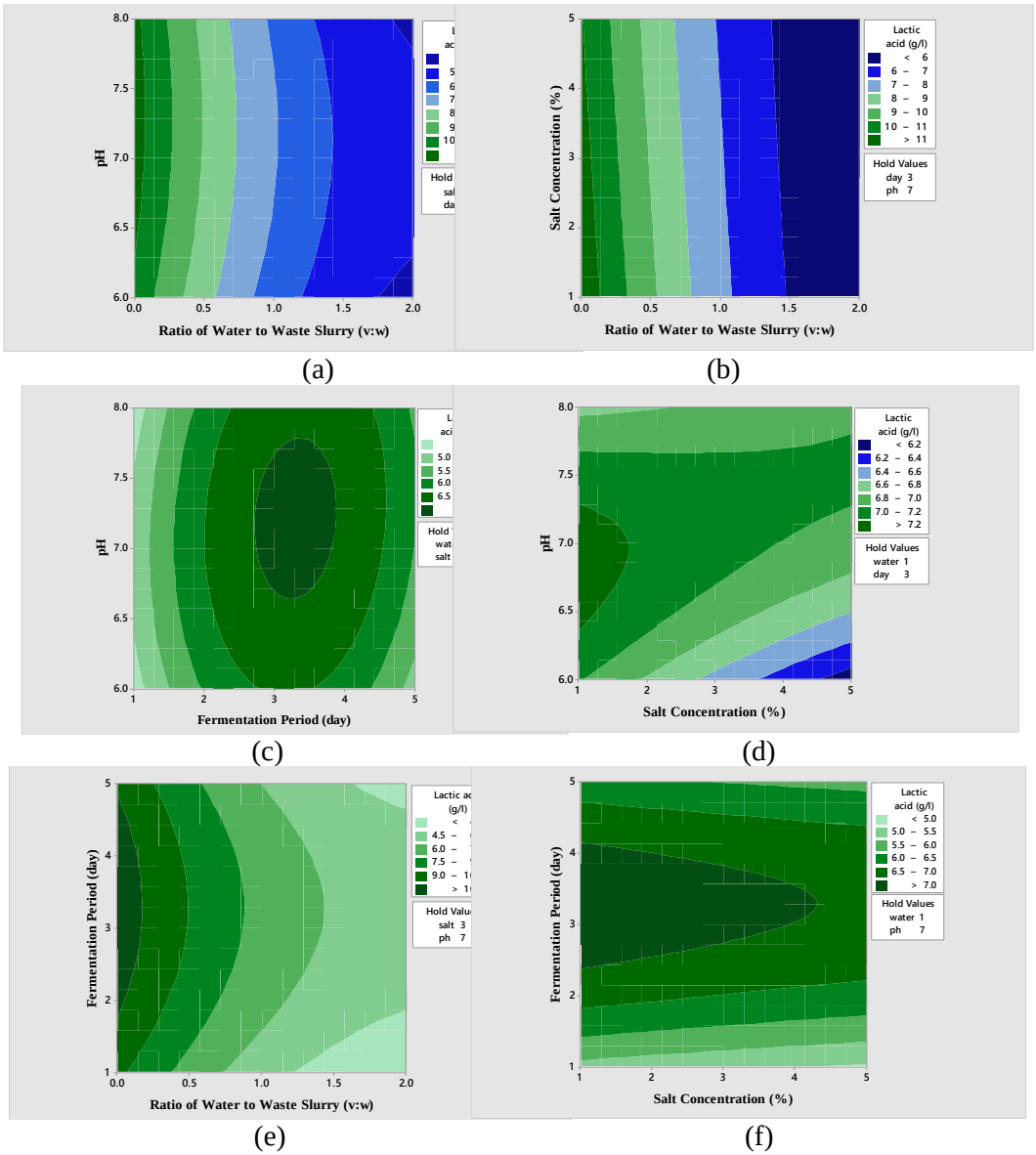


(d)



(e) (f)

Figure 1: Response Surface Plot for Production of Lactic Acid Interactive Effect of of (a) Ratio of Water to Waste Slurry and pH, (b) Ratio of Water to Waste Slurry and Salt Concentration, (c) Fermentation Period and pH, (d) Salt Concentration and pH, (e) Ratio of Water to Waste Slurry and Fermentation Period and (f) Fermentation Period and Salt Concentration



(a) (b)

(c) (d)

(e) (f)

Figure 2: Contour Plot for Production of Lactic Acid Interactive Effect of (a) Ratio of Water to Waste Slurry and pH, (b) Ratio of Water to Waste Slurry and Salt Concentration, (c) Fermentation Period and pH, (d) Salt Concentration and pH, (e) Ratio of Water to Waste Slurry and Fermentation Period and (f) Fermentation Period and Salt Concentration

3.3 Optimization of the Process Variables Using Box-Behnken Design

The 3D response surfaces are the graphical representations of the regression equation and were used to study the interaction of the variables and to locate the optimum level of each variable for maximum response. Each 3D response surface for lactic acid production represents the different combinations of two test variables at one time while keeping the other variable at its respective zero level. From the results obtained by analysis of data obtained through production of lactic acid from vegetable wastes, suitable levels of ratio of water to waste slurry, salt concentration, initial pH and fermentation period were selected for further investigation. The suggested formulations with desired level of ratio of water to waste slurry, salt concentration and incubation period with predicted score of lactic acid. The obtained responses indicate that the yield of lactic acid of 11.7844 g /l than all other formulations and also the desirability was 0.96, highest among all other formulations. Hence, the formulation with 0:1 ratio of water to waste slurry, 1% Salt concentration, 6.99 pH and 3.30 days Incubation period was selected.

3.4 Validation of the Model

To confirm the above optimum conditions, four runs with the composition (ratio of water to waste slurry = 0:1, 1% Salt concentration, 6.99 pH and fermentation period = 3.30 days) were carried out and an average value of lactic acid 12.06 g/l was obtained. This value is very close to the predicted optimum value 11.7844 g/l and hence the confirmation experiment validated the optimum conditions predicted by Box-Behnken design.

4 CONCLUSION

In this present investigation, an attempt is made to study the ability of lactic acid producing by natural bacteria from fermentation process. As an economical approach, vegetable waste (Cabbage and Cauliflower leave waste) was used in this study. Accordingly, the cost of raw materials is one of the limiting factors of the feasible economic production. It has shown that it is also need of controlling initial pH to increase in the production of lactic acid in the case of its production from vegetable waste. The experimental and predicted values of lactic acid production is compared and found to concur with one another. Based on the findings and results, the used of waste can be considered suitable to be employed as high carbon and nitrogen sources for lactic acid production.

Box-Behnken Design (BBD) is employed for the statistical analysis. RSM was used to determine the effects of four factors (ratio of water to vegetable waste, salt %, initial pH, and fermentation time) on lactic acid production from vegetable waste. Linear, quadratic and interaction effects of these variables on lactic acid production were determined. The model generated in this study by RSM satisfied all the necessary arguments for its use in optimization. By fitting the experimental data to a second-order polynomial equation, the optimum levels of the above-mentioned variables were determined. Using the optimum levels of fermentation parameters, a maximum lactic acid concentration of 12.06 g/l was obtained. This study indicates that the medium design using statistical techniques such as RSM can be very useful in improving the production of lactic acid and in similar bioprocesses.

5 ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Department of Chemical Engineering, Yangon Technological University and Food Industries Development Supporting Laboratory (FIDSL) of Myanmar Food Processors and Exporters Association (MFPEA) for supporting facilities required for this experiment.

REFERENCES

- Akerbeg, C. and Zacchi, G. (2000). An Economic Evaluation of the Fermentative Production of Lactic Acid from Wheat Flour. J. of Bioresources Technology. 75, 119-126.

- Amenaghawon N.A. Nwaru K.I. Aisien F.A. Ogbeide S.E. and Okieimen C.O. (2013). Application of Box-Behnken design for optimization of Citric acid production from corn starch using Aspergillus niger, Br. Biotechnol. 3(3), 236-245.
- Bosque-Sendra J.M. Pescarolo S. Cuadros-Rod´riguez L. García-Campana A.M. Almansa-López E.M. (2001). Optimizing analytical methods using sequential response surface methodology: Application to the pararosaniline determination of formaldehyde, Fresenius J Anal Chem. 36, 715–718.
- Coelho L.F. de Lima C.JB. Rodovalho C.M. Bernardo M.P. Contiero J. (2011). Lactic acid production by new Lactobacillus plantarum LMISM6 grown in molasses: optimization of medium composition, Braz J Chem Eng. 28(1), 27-36.
- Lunt, J. (1998). Large-scale production, properties and commercial applications of polylactic acid polymers, Polym. Degrad. Stabil., 59, 145–152.
- Sailaja Daharbha, Mohit S. Mishra, Rakesh K. Meena, Roshini Meghanani, Prashant Shukla. (2015). Optimization of Vegetable Wastes for Lactic Acid Production: A Laboratory Scale Approach. Journal of Microbiology, Biotechnology and Food Sciences, 4.5, 452-455
- Wee, Y. J., Kim, J. N., Ryu, H. W. (2006). "Biotechnological production of lactic acid and its recent applications." Food Technology and Biotechnology. 44(2), 163-172.
- Zhou, S., Shanmugam, K. T., Yomano, L. P., Grabar, T. B. and Ingram L. O. (2006). Fermentation of 12% (w/v) glucose to 1.2 M lactate by Escherichia coli strain SZ194 using mineral salts medium. Biotechnol, Lett., 28, 663-670.

Recovery and Functional Properties of Muscle Proteins Extracted from Indian Threadfin Bream (*Nemipterus Japonicus*) by pH-Shift Processing

Supriya. D. Meshre[1*], S. B. Patange[1], Rakesh R. Jadhav[2] and Sanna Shah[1]

[1]College of Fisheries, Ratnagiri, PIN-415629
[2]Diploma in Fisheries Engineering, Ratnagiri, PIN-415629
(Dr. Balasaheb Sawant Konkan Agricultural University, Dapoli) Maharashtra, India.
*Corresponding Author (supriyameshre@gmail.com)

ABSTRACT

The pH-shift processing involves the process of solubilisation of muscle proteins at high alkaline pH (11.0) and/or low acidic pH (3.0) followed by precipitation at pH (5.5) of isoelectric point. This method was used for the preparation of surimi from fish mince of Indian threadfin bream (*Nemipterus japonicus*). The study demonstrated the biochemical and functional properties of surimi prepared by acid and alkaline solubilisation. The protein recovery by alkali (76.12%) solubilisation gave better yield as compared to acid (58%). With regard to the proximate composition, highest moisture content was observed in acid surimi, highest protein was observed in alkaline surimi. Lipid and ash content were low in both the surimi samples. The protein solubility and total sulfhydryl groups were highest in alkali followed by acid surimi. The gel strength (232.34 g.cm) and whiteness of alkali surimi was observed to be significantly higher than the acid surimi. The highest expressible moisture was found in acid (7.82%) surimi, followed by alkaline (6.92%). SDS-PAGE of proteins of both the surimi samples showed band intensity of MHC at 200 kDa and actin at 45 kDa which were unaffected by pH solubilisation. The functional characteristics of alkali solubilized surimi gave better results than acid solubilized surimi by pH – shift processing.

KEYWORDS: Pink Perch, Surimi, pH-Shift Processing, Functional Properties.

1 INTRODUCTION

Fish has been important source of protein and other nutrients for human. As the global population grows and traditional fish species decline, the fish supply was growing at a slower pace compared with the demand (Hultin and Kelleher, 2000). Many fish protein resources were underutilized for human consumption such as fish processing by-products, small fatty fishes and by-catch. A major challenge facing fisheries was to find economic ways to transform these underutilized fish protein resources into human foods to meet the demand while protecting the environment from pollution (Kristinsson *et al.*, 2006). Production of mince as a new technology assumes importance when applied to low value fish (Balachandran, 2001) and by-catch that face difficulty in marketing as fresh fish or processing into conventional product.

Fish meat is minced and water-washed, and after antidenaturants was added, frozen into a block form (Suzuki, 1981). Surimi technology has been widely developed. Surimi is produced by repeatedly washing mechanically separated fish flesh with chilled water (5 °C) until most of the water-soluble proteins were removed. The washing procedure was of great importance for surimi quality – not only for removing fat and undesirable materials, such as blood, pigments and odorous substances – but, more importantly, for increasing the concentration of myofibrillar protein, thereby improving gel-forming ability (Lanier & Lee, 1992).

Proteins recovery using acid or alkali extraction followed by isoelectric precipitation provides extremely high yields (35–45%) with the inclusion of sarcoplasmic proteins and it also demonstrates better functional properties. The extraction mechanism of the two processes were to solubilise the muscle proteins at low or high pH to separate soluble proteins, bone, skin, connective tissue, cellular membranes, and neutral lipids through the centrifugation. The solubilised proteins are collected and

recovered by isoelectric precipitation to give a highly functional and stable fish protein isolate (FPI) (Kristinsson *et al.,* 2006).

Conventional surimi production is aimed to concentrate myofibrillar proteins by removing sarcoplasmic proteins, fat, blood and pigments through continuous washing of the mechanically separated fish mince (Park *et al*., 1997). A washing process can improve the functionalities and sensory characteristics of fish meat. However, a low yield is obtained when conventional washing was used (Kristinsson *et al.,* 2005). To overcome this problem, a new approach of recovering proteins by a pH-shift process, which has been developed by (Hultin and Kelleher, 2000), can be considered as a potential alternative to conventional process of surimi process.

As proteins assume a more positive or negative net charge, they gradually start electrostatic interactions with water (i.e., protein–water interactions). Due to increased protein–water interactions, the protein–protein hydrophobic interactions decrease. Therefore, as the protein molecules become more polar (charged), more water associates on and around the protein surface and the proteins become water-soluble. However, it is possible to adjust the pH of a protein solution so that the number of negative charges on a protein's surface is equal to the number of positive charges; and therefore, the protein molecule assumes a zero net electrostatic charge. The pH at which the net electrostatic charge of a protein is equal to zero is called the isoelectric point (pI) (Gehring *et al*., 2011). Using pacific whiting fillets, a conventional three-washing cycle surimi processing yielded only 40% recovery compared with 60% recovery using acid-aided processing (Choi and Park, 2002). The process also recovered the sarcoplasmic proteins and results in a higher yield recovery than the conventional method and even minimizes the use of water. In the present study, the biochemical and functional properties of fish muscle proteins prepared by using of acid and alkaline solubilization process were studied. Therefore, the purpose of this study was to investigate the protein extraction process using pH-shift and to evaluate its effect on protein characteristics.

2 MATERIALS AND METHODS

2.1 Preparation of Surimi: Acid/Alkali Solubilisation and Isoelectric Precipitation.

Fresh fishes were purchased from Mirkarwada landing centre, Ratnagiri. They were bought to College of Fisheries, Shirgoan, Ratnagiri, fishes were iced and kept below 2 ºC before processing. Mince was separated using meat mincer (BADDER 600).

The acid-alkali solubilization of protein process was carried out according to the method of Hultin and Kelleher (2000). The fish mince was homogenized (Remi motor brand homogeniser, RPM 8000) at a 1:9 (w/v) ratio with cold distilled water (4 °C). The pH of the homogenate was adjusted to 11.2 by using 2N NaOH for alkaline extraction process and 2.5 by using 2N HCl for acid. The reaction was carried out for 40 minutes with frequent stirring. The homogenate was centrifuge (Hettich Zentrifugen, D-78532, Germany) at 6,000*g* for 20 min at 4 °C. The alkaline soluble fraction was collected and adjusted to isoelectric point of muscle protein (pH 5.5) by using 2N NaOH or 2N HCl respectively. The precipitate was then filtered through cheese-cloth and was dewatered by centrifugation at 6,000*g* for 20 min at 4 °C. The final pH of the sample was adjusted to 7.0 and mixed with cryoprotectants (4% sugar and 0.2% polyphosphates). The mince was frozen at – 40 °C and stored at -20 °C.

2.2 pH Measurement

Sample (5 g) was ground with 45 ml distilled water and filtered using a filter paper. The pH of filtrate was recorded using a pH meter (AOAC, 2005).

2.3 Proximate Composition

The moisture, protein, ash and fat content were determined as per standard methods of (AOAC, 2005).

2.4 Biochemical Test and Functional Properties of Surimi.

2.4.1 Protein Recovery

Protein recovery of the washed mince from different washing methods was determined according to the method of Kim et al. (2003). The recovery was expressed as the weight of recovered protein divided by the weight of the minced fish (at the same moisture content). After an acid-aided, alkaline-aided or conventional washing process, the moisture content of washed mince and protein isolates was equally adjusted to 79% moisture (the initial moisture content of fish muscle); the weight of recovered protein at the same moisture content was recorded. The recovery of protein was calculated as follows:

$$\text{Protein recovery (\%)} = \frac{\text{weight of recovered washed mince (g)}}{\text{weight of initial minced sample (g)}} \times 100 \quad (1)$$

2.4.2 Protein Solubility

Protein solubility was determined using method of Choi and Park. (2002). Samples (2g) were homogenized with 18 ml of 0.5M borate buffer solution pH 11.0 for 1 minute and stirred for 30 min at 4 °C. The homogenates were centrifuged at 8000*g* for 5 min at 4 °C and the protein concentration of the supernatant was measured by the Biuret method. Protein solubility (%) was defined as the fraction of the protein remaining soluble after centrifugation and calculated as follows:

$$\text{Protein solubility (\%)} = \frac{\text{protein concentration in supernatant}}{\text{protein concentration in homogenate}} \times 100 \quad (2)$$

2.4.3 Total Sulfhydryl (SH) Groups

The total SH groups of myofibrillar protein fraction were estimated according to Sedlak and Lindsay (1968). A 400 mg of sample (NAM) was homogenate with 8.0ml of 0.02M EDTA in ice bath. Aliquots of 0.5 ml of the homogenate were mixed in 15 ml test tube with 1.5 ml of 0.2 M Tris buffer (pH 8.2) and 0.1 ml of 0.01 M 5,5'-dinitrobis-(2-nitrobenzoic acid) (DTNB). The mixture was brought to 10 ml with 7.9 ml of absolute methanol. A reagent blank and sample blank (without DTNB) were prepared in a similar manner. The test tubes were stopper with rubber caps and allowed to stand with occasional shaking for 30 min, then filtered twice using Whatman No.1 filter paper producing a clear filtrate. In an alternate procedure, colour was developed for 15 min and the reaction mixture centrifuged at 3000*g* at room temperature for 15 min. The absorbance of the supernatant was read at 412 nm using spectrophotometer. The values of total SH groups for the samples were calculated using standard curve plot of different concentrations of cysteine. The sulfhydryl groups were expressed as μ moles of total sulfhydryl groups / g protein.

2.4.4 Surimi Gel Preparation

The heat-induced surimi gel was prepared according to Kunimoto et al. (2016) prepare the gel, frozen mince, was thaw for 30 min in running water (26-28 °C) until the core temperature reached 0^0 C, the samples were cut into small pieces (1 cm thickness) and moisture content was adjusted to 80 %. Dry Sodium chloride was added to the sample (2.5%, w/w) and mince was chopped for 4 min at 4 °C to obtain homogeneous sol. The sol was then stuffed into polyvinylidine casing with diameter of 2.5 cm and both ends of casing were sealed tightly. The sol was then incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a water bath. The gels were cooled in iced water and stored for 24 hours at 4 °C prior to analysis.

2.4.5 Gel Strength of Surimi Gel

The gel strength of surimi gel was measured by a texture analyser. Chill stored gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared from each gel. The breaking force (gel strength) and deformation (elasticity or cohesiveness)

were measured by using the texture analyser equipped with a cylindrical plunger (5 mm diameter; 60 mm per min penetration speed). Measurements were taken in triplicates.

2.4.6 Whiteness Measurement of Surimi Gel

Whiteness of surimi gels was measured using a (Hunterlab Miniscan EZ Model No. 4500L) method described by Rakesh and Anil (2018). The measurement of L^*(lightness), a^* (redness/green) and b^* (yellowness/blueness) was performed in five replications. Whiteness was calculated using the following equation (Lanier *et al.,* 1991).

$$\text{Whiteness} = 100 - [(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2} \quad (3)$$

2.4.7 Expressible Moisture of Surimi Gel

Expressible moisture content of surimi gel samples was measured according to the method of Benjakul *et al.,* (2001) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed (X) and placed between 3 pieces of Whatman paper No. 4 at the bottom and 2 pieces on the top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The samples were then removed from the papers and weighed again (Y). Expressible moisture content was calculated using the following equation:

$$\text{Expressible moisture content (\%)} = 100[(X-Y)/X] \quad (4)$$

2.4.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE*)*

Electrophoretic analysis of protein using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) using 5% stacking gel and 8% separating gel. Sample (3g) was homogenized with 27 ml of 5% (w/v) SDS for 1 minute at a speed of 11,000 rpm. The homogenate was incubated at 85 °C for 1 hour to dissolve total proteins, followed by centrifugation at 3500 *g* for 20 minutes at room temperature, to remove undissolved debris. The 20 µl supernatant were mixed with 5 µl sample loading buffer and boiled the tubes containing protein sample at 100 °C for 3 minutes in a boiling water bath. The sample (20 µl protein) was loaded into polyacrylamide gel and subjected to electrophoresis at a constant current of 110 volts. After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 and distained with solution containing 50% distilled water (v/v), 40% (v/v) methanol, and 10% (v/v) acetic acid.

2.5 Statistical Analysis

All analyses were carried out in triplicates and data expressed as means ± standard deviations. Analysis of variance (ANOVA) were carried out to assess significant differences between means ($p<0.05$).

3 RESULTS AND DISCUSSION

The recovery/yield of proteins of pink perch muscle by alkaline and acid methods was 76.12%, 58.54%; recovery of alkaline method was highest as compared to acid (Table 1).

Table 1: Protein recovery by alkaline and acid solubilisation method.

Surimi	pH of protein solubilisation	Protein recovery (%)
Alkaline surimi	11.0	76.12
Acid surimi	3.0	58

In the comparison between acid and alkaline method, Kristinsson and Ingadottir (2006) investigated that higher protein recovery was 61% to 68% by alkaline pH (11.0) method as compared to acid pH (2.0) method with 56% to 61% yield from tilapia (*O. niloticus*). Batista (1999) found the protein yield of 80.6% and 62.9% by alkaline method after the isoelectric precipitation of hake (*Merluccius merluccius*) and monkfish (*Lophius piscatorius*) respectively, modifying slight difference in processes of Hultin and Kelleher, some salts was added and extend the extraction time and higher temperatures were used. As the majority of food proteins are acidic (pH > 7), they have minimum solubility at isoelectric pH (4.0-5.0) and maximum solubility at alkaline pH. The minimum solubility at isoelectric pH was mainly due to lack of electrostatic repulsion which promotes aggregation and precipitation via hydrophobic attractions. The observation made with present study regard to yield proteins was in agreement with Kristinsson and Ingadottir (2006) and Batista (1999). The higher recovery of protein was obtained from alkaline method and lower from acid method was due to precipitation of sarcoplasmic protein as well.

The moisture content of alkaline and acid surimi was 79.06 ± 2.43%, 80.20 ± 0.49% (Table 2). While the protein content was highest alkaline surimi 18.22 ± 0.02% followed by acid surimi 17.71 ± 0.32%. The fat and ash content were found low in both the surimi samples Table 2.

Table 2: Proximate composition of alkaline and acid surimi

Surimi	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Alkaline Surimi	79.06±2.43	18.22±0.02	0.52±0.03	2.20±0.22
Acid Surimi	80.20±0.49	17.71±0.32	1.03±0.01	1.38±0.03

The result shows that the moisture content was increased in acid surimi which is due to the charged group (ion-dipole interaction) during acid extraction process. Undeland *et al.* (2002) found that protein isolates samples that were prepared from alkaline method were lower in fat than the acid method. This means that in preparation of protein isolated by the alkaline method, it enable removal of fats. The ash content in acid surimi was lower than the alkaline surimi. Marmon and Undeland, (2010) reported that the amount of ash should be seen as a measure of impurities, and it is of great importance to reduce it to concentrate the proteins.

The higher protein solubility values were observed in alkaline surimi 64.43% followed by acid surimi 60.92% (Fig 1). Protein solubility in fish muscle has been used as a criterion for the alteration of proteins and decrease in protein solubility, as a result of protein denaturation, subsequently increased hydrophobic interactions, which caused precipitation of the protein (Zayas, 1997). The lower solubility was due to more improperly unfolded protein (Kristinsson and Hultin, 2004). Among the surimi, the alkaline processed surimi has higher protein solubility than the acid processed.

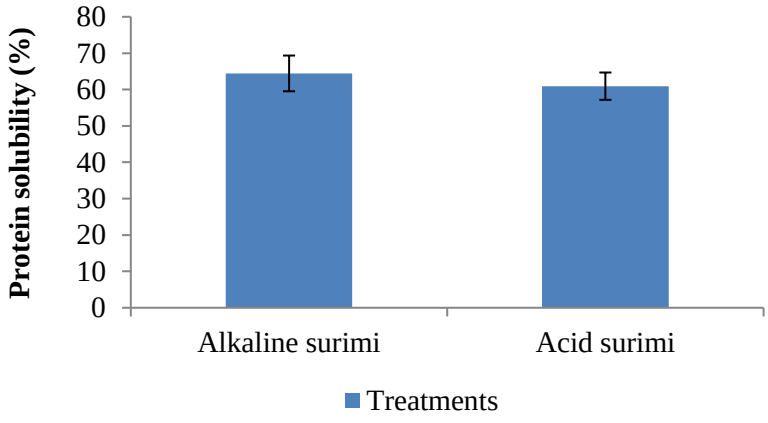


Figure 1: Protein solubility (%) of pink perch surimi prepared by alkaline and acid solubilization method. Note: Error bars indicate the SD

Total sulfhydryl content was observed in both the surimi samples, the highest was found acid surimi followed by alkaline surimi (Fig 2). Reduction in the total sulfhydryl content is due to the formation of disulfide bonds or oxidation of sulfhydryl groups or exchange of disulfide bond (Benjakul *et al.,* 1997). Yogsawatdigul and Park (2004) reported decrease in SH groups in acid treated samples, this suggested that oxidation and SH/S-S interchange reactions could occur during acid solubilization.

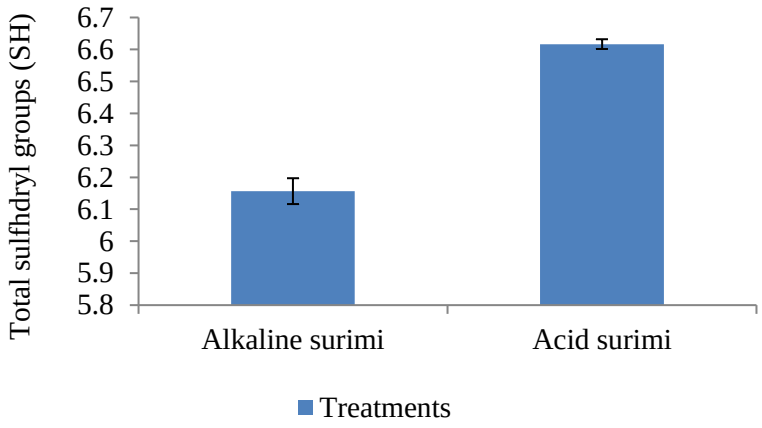


Figure 2: Total sulfhydryl content of pink perch surimi prepared by alkaline and acid solubilization method. Note: Error bars indicate the SD

The gel prepared by the alkaline surimi showed higher values than the acid surimi (Fig 3). Yongsawatdigul and Park (2001) demonstrated that rockfish protein isolates produced from alkali-aided process had better gel-forming ability as compared to the acid-aided and conventional surimi processes. Ninomiya *et al.,* (1990) also observed the formation of more preferable gels when fish muscles treated with pH 12.0 than pH 3.0 using water soluble proteins from mackerel. The result suggested that due to pH shift processing in acid condition the fish protein undergoes extreme denaturation.

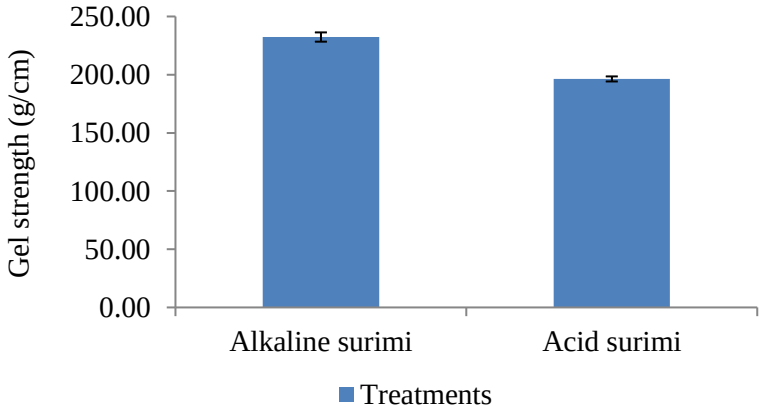


Figure 3: Gel strength of pink perch surimi prepared by alkaline and acid solubilization method. Note: Error bars indicate the SD

The whiteness of surimi gel prepared alkaline and acid surimi were recorded as 74.51, 58.70 (Table 3). The whiteness of acid surimi gel was lower than the alkaline surimi gel due to the presences of myoglobin in the muscle, skin pigments, haemoglobin etc. The decrease in whiteness of acid surimi was probably due to the adduction of pigment protein, especially oxidised pigment to muscle proteins. The result shows that higher whiteness in alkaline surimi was may be due to the heat treatment and changes of native heme protein during protein gelation. The lowest whiteness in acid surimi was probably due to higher levels of denatured and oxidised heme proteins.

Table 3: whiteness and expressible moisture content of pink perch surimi prepared by alkaline and acid solubilisation method.

Treatment	Whiteness		Expressible moisture content (%)
Alkaline surimi	L*	77.37 ± 0.58	6.92 ± 0.03
	a*	-2.43 ± 0.72	
	b*	11.46 ± 0.48	
	Whiteness	74.51 ± 0.54	
Acid surimi	L*	66.97 ± 0.96	7.82 ± 0.04
	a*	0.17 ± 1.23	
	b*	24.79 ± 7.56	
	Whiteness	58.70 ± 4.01	

The results of the present study show that expressible moisture content (%) of alkaline surimi gel 6.92% was lower than the acid surimi gel 7.82% (Table 3). The lowest expressible moisture content was found in alkaline surimi which indicates that the protein network of that gel was higher in water-holding properties. The higher expressible moisture in surimi gel was found in gel samples prepared acid surimi; which was due to the poor gel network of pH-shift process. In addition, adjusting the pH of protein isolate to neutral can enhance the renaturation of proteins to some extent and alter the water-binding property of protein (Rawdkuen *et al.*, 2009).

As can be seen in (Fig 4) of electrophoresis of proteins of both the surimi samples observed in SDS-PAGE, the band intensity of the heavy chain of myosin was at 210 kDa and actin 45 kDa were observed. The SDS-PAGE protein with molecular weight of 245, 180, 135, 100, 75, 63, 48, 35 kDa were observed in the samples. Choi and Park (2002) found cathepsin L activity in Pacific whiting muscle proteins treated by the acid solubilization process. And they noted that both acidic and alkaline solubilization processes did not significantly promote proteolysis of myosin heavy chain myosin heavy chain (MHC) as indicated by a minimal loss of MHC in both samples. Yeung and Jinx-Soo (2005) suggested that degradation of MHC in croaker and jack mackerel, under acidic conditions, was higher than that under alkaline conditions. Decrease of MHC in gels with the acid surimi was coincidental with decrease in the gel strength. However, it was not clear whether the reduction of myosin heavy chain resulted from the degradation of myosin by acid protease or acid hydrolysis. From the results it was showed that more MHC retained in alkaline processed surimi.

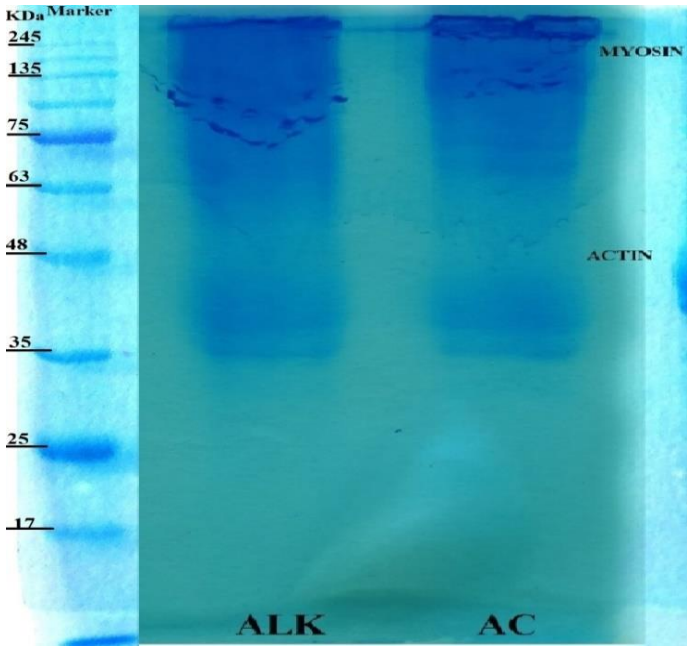


Figure 4: SDS – PAGE of pink perch surimi prepared by alkaline and acid solubilisation method. Lane 1: Marker, ALK: Alkaline surimi, AC: Acid surimi.

4 CONCLUSION

From the study it was concluded that the higher protein recovery/yield was achieved from alkaline processed surimi. The higher gel strength and whiteness was observed in alkaline surimi. The acid surimi having the higher expressible moisture content that results in low water-binding property. The pH-shift processing method can be used to recover the protein and the final product the fish protein isolates with conventional surimi can used to prepared value added product.

5 ACKNOWLEDGEMENTS

This paper forms the part of M.F.sc (Maters in Fisheries Science) research work of Supriya Meshre. The authors are thankful to Associate Dean, College of Fisheries, Shirgaon, Ratnagiri, India.

REFERENCES

- AOAC, (2005). Official methods of analysis (17th ed.). Washington, DC: Association of official Analysis Chemists.
- Balchandran, K. K. (2001). *Post-harvest Technology of fish and fish products*. Daya publication house Delhi, pg. 271.
- Batista, I. (1999). Recovery of proteins from fish waste products by alkaline extraction. European Food Research and Technology, 210, 84–89.
- Benjakul S., Visessanguan, W., & Srivilai, C. (2001). Gel properties of bigeye snapper (*Priacanthus tayenus*) surimi as affected by setting and porcine plasma protein. Journal of Food Quality, 24, 453–471.
- Benjakul, S., Seymour TA, Morrissey MT, An H. (1997). Physicochemical changes in Pacific whiting muscle proteins during iced storage. Journal of Food Science, 62,729-733.
- Choi, Y. J., & Park, J. W. (2002). Acid-aided protein recovery from enzyme-rich Pacific whiting. Journal of Food Science, 67(8), 2962–2967.
- Gehring, C. K., Gigliotti, J. C., Moritz, J. S., Tou, J. C., Jaczynski, J. (2011). Functional and nutritional characteristics of proteins and lipids recovered by isoelectric processing of fish by-products and low-value fish: A review. Journal of Food chemistry, 124, 422-431.
- Hultin H.O, Kelleher, S.D (2000). High efficiency alkaline protein extraction. U.S. patent 6, 136, 959. University of Massachusetts, Oct. 24, 2000.
- Kim, Y.S., Park, J.W., & Choi, Y.J. (2003). New approaches for the effective recovery of fish proteins and their physiochemical characteristics. Fisheries science, 69, 1231-1239.
- Kristinsson, H., & Hultin, H. O. (2004). The effect of acid and alkali unfolding and subsequent refolding on the pro-oxidative activity of trout hemoglobin. Journal of Agricultural and Food Chemistry, 52, 5482–5490.
- Kristinsson, H., & Liang, Y. (2006). Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*) muscle proteins. Journal of Food Science, 71(5), C304–C312.
- Kristinsson, H., Theodore, A. E., Demir, N., & Ingadottir, B. (2005). A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from Channel catfish muscle. Journal of Food Science, 70(4), C298–C306.
- Kristinsson, H.G., Ingadottir B. (2006). Recovery and properties of muscle proteins extracted from tilapia (*Oreochromis niloticus*) light muscle by pH shift processing. Journal of Food Science, 71,E132–41.
- Kunimoto, M., Hamada-Sato, N. and Kato, N. (2016). Main protein components in frozen surimi contributed to heat-induced gel formation. International Food Research Journal 23(5), 1939-1948.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680-685.
- Lanier, T.C., and Lee, C. M. (1992). Surimi technology. New York: Marcel Dekker.

- Lanier, T.C., Hart, K. and Martin, R.E. (1991). A Manual of standard methods for measuring and specifying the properties of surimi. Washington D.C.: National Fisheries Institute.
- Marmon, S., and Undeland, I. (2010). Protein Isolation from Gutted Herring (*Clupea harengus*) Using pH-Shift Processes. Journal of Agriculture Food Chemistry, 58, 10480–10486
- Ninomiya K, Ookawa T, Tsuchiya T, Matsumoto JJ. (1990). Concentration of fish waste soluble protein and its gelation properties. Nippon Suisan Gakkaishi 56:1641-1645.
- Park, J. W., Lin, T. M., & Yongsawatdigul, J. (1997). New developments in manufacturing of surimi and surimi seafood. Food Review International, 13, 577–610.
- Rakesh, J and Anil, A. (2018). Experimental investigation on biochemical, microbial and sensory properties of Nile tilapia (Oreochromis niloticus) treated with moringa (Moringa oleifera) leaves powder. Journal of Food Science and Technology, 55(9):3647–3656.
- Rawdkuen, S., Sai-Ut, S.,Khamsorn, S., Chaijan, M. and Benjakul, S. (2009). Biochemical and gelling properties of tilapia surimi and protein recovered by acid-alkaline process. Journal of food chemistry, 112, 112-119.
- Sedlak. J and Raymond H. Lindsay. (1968). Estimation of Total, Protein-Bound, and Nonprotein Sulfhydryl Groups in Tissue with Ellman's Reagent. Analytical biochemistry 25, 192-205.

  Suzuki. T. (1981). Fish and krill protein processing technology. Applied science publisher ltd, London, Pg. 115.
- Undeland, I., Kelleher, S. D., & Hultin, H. O. (2002). Recovery of functional proteins from herring (*Clupea harengus*) light muscle by an acid or alkaline solubilization process. Journal of Agricultural and Food Chemistry, 50(25), 7371–7379.
- Yeung, C. J., & Jinx-Soo, K. (2005). Fish protein recovered using pH shifting method and its physicochemical properties. Journal of Ocean University of China, 4, 224–228.
- Yongsawatdigul ,J., Park, J.W. (2001). Gelation characteristics of alkaline and acid solubilization of fish muscle proteins [abstract]. In: IFT Annual Meeting Book of Abstracts; 2001 June 23-27; New Orleans, La. Chicago, Ill: Institute of Food Technologists. P 251. Abstract nr 100 1.
- Yongsawatdigul, J., & Park, J. W. (2004). Effects of alkali and acid solubilization on gelation characteristics of rockfish muscle proteins. Journal of Food Science, 69(7), 499–505.
- Zayas, J. F. (1997). Functionality of proteins in food. Berlin: Spring-Verlag. Yongsawatdigul, J., & Park, J. W. (2004). Effects of alkali and acid solubilization on gelation characteristics of rockfish muscle proteins. Journal of Food Science, 69(7), 499–505.

Effect of Growing Culture on Growth Characteristics of *Acetobacter Pasteurianus* NH6 and Their Survival after Heat-Pump Drying

Hien Nguyen Minh and Huan Phan Tai*

Faculty of Food Science and Technology, Nong Lam University- Ho Chi Minh City, Vietnam
*Corresponding Author (pthuan@hcmuaf.edu.vn)

ABSTRACT

Used as starter cultures, *Acetobacter pasteurianus* NH6 isolated from fermentation of cocoa beans were cultured in different media and subjected to heat-pump drying. The study aimed to determine the effect of the growing cultures (comprised of AAM medium, potato extract, coconut water and cocoa pulp juice) on the growth characteristics of the studied bacteria. Screening of different carrier agents (maltodextrin, starch acetate, corn and soybean flours) subjected to heat-pump drying of *A. pasteurianus* NH6 grown at 30ºC, pH 5.0 -5.5 and shaking at 150 rpm in 48 hours. A mixture of corn and soybean flours (4:1; w/w) was confirmed as a carrier agent during heat-pump drying at 45ºC in 5.1 hours. A drying recovery of 99.07% was found with a survival rate of *Acetobacter pasteurianus* NH6 at 77.64%. The viability of these bacteria in the product of heat-pump drying was obtained at 8.43 log cfu/g.

KEYWORDS: *Acetobacter pasteurianus* NH6, growing media, heat-pump drying, carrier agent, survial rate.

1 INTRODUCTION

Yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) grow during cocoa bean fermentation. In the initial phase, the yeasts produce ethanol by fermenting sugar under anaerobic conditions. LAB become dominant after 12–24 hours of fermentation, convert sugar mainly into lactic acid. After a mixing of cocoa bean mass, as more air penetrate, AAB start to grow and involve in oxidizing the ethanol (produced by the yeasts) and lactic acid (produced by the LAB) into acetic acid. AAB has been reported to play a role in the fermentation of cocoa bean in previous studies (Forsyth, 1963; Schwan, 2004; Lima *et al.*, 2011). Acetic acid which has been produced by ABB, diffuse into the beans, in combination with the heat produced by this exothermic bioconversion, cause the death of seed embryo as well as the end of fermentation and cause production of flavor precursors (Camu *et al.*, 2007). AAB, mainly *Acetobacter* was isolated and displayed maximum development after 48 hours (1.5×10^8 CFU g^{-1} of dry matter) (Sandra *et al.*, 2007). Beside *Acetobacter pasteurianus* which was the predominant AAB in all investigated cocoa bean fermentations, different *Acetobacter* species may be present such as *A. syzygii*, *A. tropicalis* (Nielsen *et al.*, 2007; Camu *et al.*, 2007), *A. ghanensis*, *A. senegalensis* (Philipp *et al.*, 2014).

The aim of this study was to determine the effect of the growing cultures on the growth characteristics of *A. pasteurianus* NH6; screening of different carrier agents subjected to heat-pump drying toward development starter culture for cocoa bean fermentation.

2 MATERIALS AND METHODS

2.1 Microorganism:

Acetobacter pasteurianus NH6 was screened out of 9 natural isolates from cocoa bean fermentation (Vu *et al.*, 2017)

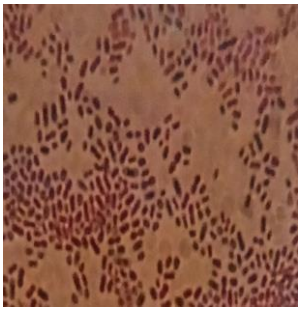
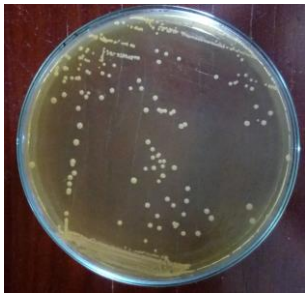



Figure 1: Cell and colonies of *Acetobacter pasteurianus* NH6

2.2 The cocoa pulp juice

Cocoa juice was collected in Nguyen Loc Co. Ltd, Dong Nai province, Vietnam. After the pods were opened, seeds were pressed to collect juice from mucilage of cocoa beans (10% of bean weight). The juice was obtained at pH 3.75±0.01; 23±2.7 ^{o}Brix; 18.3±2.08% total sugar and 12.7±2.03 g/100ml reducing sugar.

2.3 Media Culture

Acetic acid medium (AAM) comprised 1% D-glucose, 0.5% ethanol, 0.3% acetic acid, 1.5% peptone, 0.8% yeast extract (Ilse *et al.* 2008).

The medium of potatoes extract included 11% potato dextrose broth (Difico), 0.3% peptone; 0.5% yeast extract; 0.5% ethanol; 0.03% acetic acid; pH4.2 (Lu *et al.*, 1999).

The medium of coconut water was obtained with 20% glucose; 2% $(NH_4)_2SO_4$; 2% KH_2PO_4; 2% $MgSO_4.7H_2O$; 1 lit coconut water; pH 4.0 – 4.5 (Đinh *et al.*, 2007).

The medium of cocoa pulp juice 1 comprised 50% cocoa juice, 0.5% ethanol, pH 4.0 - 4.5. The medium of cocoa pulp juice 2 included 50% cocoa pulp juice; 0.5% yeast extract; 0.3% pepton; 0.5% ethanol; pH 4.0 – 4.5.

The medium of cocoa pulp juice 3 was obtained with 50% cocoa pulp juice; 0.5% yeast extract; 0.3% pepton; 0.5% ethanol; pH 5.0 – 5.5. All the medium cultures were autoclaved at 115^{o}C/ 15 minutes.

The objective of this experiment was to optimize the growth conditions for *A. pasteurianus* NH6 in a simple and low-cost medium. AAM was using as the reference medium. The inoculum was always 1% (v/v) in the 6 investigated cultures; agitation of 150 rpm for 4 days at 30^{o}C. The cell growth was enumerated every day by counting viable colonies using standard dilution plate count on GYC Agar (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar, pH 5.4) and expressed in Log cfu/ml.

2.4 Carrier Agents

Carrier agents included maltodextrin DE 12; acetate starch, corn and soybean flours. 100 ml of cell broth were harvested at stationary phase and centrifuged at 6000rpm for 10 minutes. The cell was washed with 0.001M sodium phosphate buffer solution (Cardona, 2002). Different carrier agents were added to bacteria biomass including 10% maltodextrin (Linder *et al.*, 1995), 10% acetate starch; 250% corn and soybean flours (Nguyen, 2010), then dispensed into sterile glass petri, spread 1cm thin. The chamber surfaces were sprayed with 95% ethanol to minimize microbial contamination. The equipment was started 1h before use to stabilize the temperature (45^{o}C) and relative humidity of the chamber. The average air velocity inside the drying chamber was 1m/s. The drying process has lasted until 13% moisture of the samples were obtained. Both the sample and dry mass of the cells were determined by serial dilution, spreading 0.1 ml on GYC agar.

The colonies were counted and viability was determined as followed.

Viability (%)= Cf / Ci x 100 (1)

Where Cf: viable cell counts of dried sample (cfu/g)
Ci: viable cell counts of the fresh sample (cfu/ml)

3 RESULTS AND DISCUSSION

3.1 Effect of Cultures on the Growth Characteristics of *A. pasteurianus* NH6

A comparison of ability to culture *A. pasteurianus* NH6 in different media was investigated. The results of cell viability are shown in Table 1.

Table 1: Effect of cultures on the viable cells of *A. pasteurianus* NH6

Exp	Cultures	The results of cell viability (log cfu/ml)				
		Day 0	Day 1	Day 2	Day 3	Day 4
Exp 1	AAM	4^{j}	7.63^{h}	7.98^{defg}	8.29^{ab}	8.12^{bcde}
Exp 2	Potato extract	4^{j}	7.46^{h}	8.06^{def}	8.36^{a}	8.15^{bcd}
Exp 3	Coconut water	4^{j}	7.24^{i}	7.84^{g}	7.86^{g}	7.55^{h}
Exp 4	Cocoa pulp juice 1	4^{j}	7.5^{h}	7.97^{defg}	8.13^{bcde}	7.61^{h}
Exp 5	Cocoa pulp juice 2	4^{j}	7.56^{h}	7.93^{fg}	8.38^{a}	7.96^{efg}
Exp 6	Cocoa pulp juice 3	4^{j}	8.02^{defg}	8.26^{abc}	8.42^{a}	8.1^{cdef}

Cocoa pulp juice 1: 50% cocoa juice, 0.5% ethanol, pH 4.0 – 4.5.
Cocoa pulp juice 2: 50% cocoa juice, 0.5% yeast extract, 0.3% pepton, 0.5% ethanol, pH 4.0 –4.5
Cocoa pulp juice 3: 50% cocoa juice, 0.5% yeast extract, 0.3% pepton, 0.5% ethanol, pH 5.0 –5.5

Culture in experiment 1 (Exp 1) was used as reference media. There was a significant difference at confidence level 95% in the visible cells among investigated media. For 4 days, the growth of *A. pasteurianus* NH6 in the Exp 2 and Exp1 was lower while in Exp 2 and Exp 5 was similar to Exp1 (the reference media). Exp 5 and Exp 6 used the same nutrient, but difference pH. However, the cells in Exp 6 was obtained highest. As reported by Anvoh, the cocoa pulp juice was rich in glucose (214.2±6.2g/L), importance minerals such as potassium (950±16.32mg/L), calcium (171.5±34.1mg/L); ascorbic acid (18.3±7.5mg/L), citric acid (9.1±0.6mg/L), malic acid (3.6±0.5mg/L) and acetic acid (2.28±0.7mg/L). It was lower in fumaric acid, oxalic and lactic acid and also sodium, magnesium and phosphorus (Anvoh *et al.*, 2009). The explanation conceivably was due to the abundant of nutrient; pH factor also might involve in the bacteria growth. Thus, pH 5.0 – 5.5 was optimal pH for *A. pasteurianus* NH6

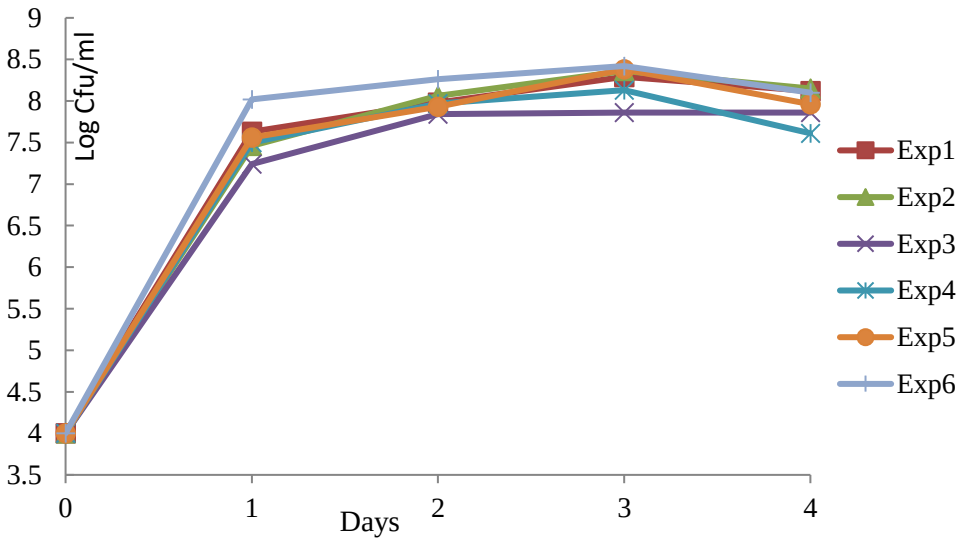


Figure 2: Effect of cultures on the growth curve of *A. pasteurianus* NH6

Growth curves were observed in Figure 2. Time was the significant effected on bacteria growth. *A. pasteurianus* NH6 was activated in enrichment media before inoculating in investigated media. In addition, cultures were abundant of nutrient. AAB grew so fast that the lag phase was absent after 24 hours in all of the cultures. Maybe, it was occurred at the previous time. The log phase, stationary phase, death phase was observed in the first and second day; the third day and the last day, respectively. The cell concentration on the second day and the third day in Exp 6 was the highest and similar ($p<0.05$). So, the biomass of *A. pasteurianus* NH6 was harvested after 48 hours of incubating (stationary phase) for further experiment. It also indicated that drying of cultures collected from stationary phase could result in better survival than those collected from log phase and other phases in the growth curve (Lavari *et al.*, 2015).

3.2 Effect of Carrier Agents on the Survival of *Acetobacter pasteurianus* NH6 in During Heat-Pump Drying*:*

The main advantages of using heat pump technology are energy saving potential and the ability to control drying temperature and air humidity. The investigated experiment was carried out to determine the time required to obtain 13 -14% moisture of dried sample (Table 2).

Table 2. Required drying time and survival rate of dried sample

Carrier agents	Time (h)	Survival rate (%)
Maltodextrin	10.5 – 11	54.90
Acetate starch	4.7 – 5	5.01
Corn flours: soybean flours (4:1)	4.8 – 5.5	77.64

Besides, incorporating dehydration – protection into dry medium could so greatly increase survival ratio by minimizing cell injury during the process (Khem, 2015). The carrier agents have significant effect on drying time, survival rate, drying recovery of *A. pasteurianus* NH6. Freeze drying and spray drying of *Lactobacillus fermentum* with maltodextrin as a carrier and a cryoprotectant may be cost effective. Maximum survivability of *L. fermentum* in freeze dried and in spray dried with maltodextrin was achieved at 89% and 86.5%, respectively (Arenahalli *et al.* , 2011). The optimal values of the variables for maximum survival and minimum moisture content of *B. bifidum* powder were as follows: inlet air temperature of 111.15°C, air pressure of 4.5 bar and maltodextrin concentration of 6%. Under optimum conditions of spray drying, the maximum survival of 28.38% was achieved while moisture was maintained at 4.05% (Zahra *et al.*, 2015). However, maltodextrin carrier gave both lowest survival of *A. pasteurianus* NH6 (5.01%) and a low recovery rate (65,45%) in heat pump drying. It requires a long time drying (10.89 hours) and maltodextrin was stick on the surface of petri due to died cell and the low recovery.

The mixture of corn flour and soybean flour (4:1, w/w) gave the highest viabilities (77.76%) and recovery (99.07%). Our result was agreed with the report by Nguyen (2010) who found that these materials were the best carrier for LAB. Living cells and biologically active molecules are generally sensitive to temperature. Heat pump can operate at milder temperature, closer to the atmospheric condition which the microorganisms naturally inhabit and thus reduces loss in biological activity caused by abrupt temperature change (Strongmen *et al.*, 1993). Heat pump dryer has been used to successfully preserve *L. lactic* sp *latis* at optimum temperature of 25^{o}/6 hours 40 minutes to achieve maximum cell survival of 92.8% when 15% dried yeast and 10% trehalose are added as protectants (Cardona *et al.*, 2002). Our investigated temperature was higher than, thus cell survival was lower.

4 CONCLUSION

The study showed that cocoa pulp (pH 5.0 – 5.5) was the good substrate for culturing *Acetobacter pasteurianus* NH6. Harvesting cell after 48 hours was suitable to prepare drying. A mixture of corn and soybean flours (4:1; w/w) was confirmed as a good carrier agent during heat-pump drying at 45^{o}C in 5.1 hours. A drying recovery of 99.07% was found with a survival rate of *Acetobacter pasteurianus* NH6 at 77.64%. The viability of these bacteria in the product of heat-pump drying was obtained at 8.43 log cfu/g. It can be used as a starter culture for fermentation of bean cocoa.

4 ACKNOWLEDGEMENT

This work was supported by Department of Sience and Technology Dong Nai Province, Vietnam.

REFERENCES

- Arenahalli Ningegowda Madhu, Sharda Prasad Awasthi, Kanchi Bhasker Praveen Kumar Reddy and Siddalingaiya Gurudutt Prapulla. (2011). Impact of freeze and spray drying on the retention of probiotic properties of Lactobacillus fermentum: An in vitro evaluation model, *International Journal of Microbiological Research 2 (3)*: 243-251.
- Anvoh, K. Y. B., Zoro Bi, A., and Gnakri, D. (2009). Production and character-ization of juice from mucilage of cocoa beans and its transformation into marmalade. *Pakistan Journal of Nutrition, 8*:129–133.
- Camu, N. , T. De Winter, K. Verbrugghe, I. Cleenwerck, P. Vandamme, J.S. Takrama, M. Vancanneyt, and L. De Vuyst. (2007). Dynamics and biodiversity of populations of lactic acid bacteria andacetic acid bacteria involved in spontaneous heap fermentation of cocoa bean in Ghana. *Appl. Environ. Microbiol. 73*:1809-1824
- Cardona, T.D.; Driscoll, R.H.; Paterson, JL.; Srzedmicki and Kim (2002) Optimizing conditions for heat pump dehydrattiong of lactic acid bacteria, *Drying Technology, 2002 (8),* 1611-1632
- Nielsen D.S.; O.D.Teniola; L.Ban-Koffi; M.Owusu; T.S.Andersson; W.H.Holzapfel. (2007) The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods, *Internatinal journal of food microbiology, Volume 111, Issue 2*, pp. 168 – 186
- Đinh Thị Kim Nhung, Nguyễn Thi Thùy Vân, 2012, Tối ưu hóa điều kiện nuôi cấy cho vi khuẩn *Acetobacter xylinum* D9, *Tạp chí sinh học, 34(3*): 337-342 (In Vietnamese)
- Forsyth, W.G.C., Quesnel, V.C. (1963). Mechanisms of cocoa curing. *Advances in Enzymology, 25*: 457–492.
- Ilse Cleenwerck , Ángel Gonzalez , Nicholas Camu , Katrien Engelbeen , Paul De Vos, Luc De Vuyst. *(*2008). Acetobacter fabarum sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation, *International jounal of systematic and evolutionary microbiology, 58*, 2180-2185.
- Khem, S.; Woo, M.; Small, D.M, Chem, X. D and May, B. K. (2015). Agent selection and protective effects during single droplet drying of bacteria. *Food chemistry, 166*, 206-214.
- Lavari, L.; Paez, R.; Zotta, T.; Cuatrin, A; Reinheimer, J; Parente, E. And Vinderoa, G. (2015). Growth of *Lactobacillus rhamnosus* 64 in whey permeate and study of the effect of mid stresses on survival to spray drying, *Food Science and technology, 63 (1),* 322- 330.
- Lima, L. J. R., Almeida, M. H., Nout, M. J. R., and Zwietering, M. H. (2011). Thobroma ca cao L., "The food of the gods": Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. *Critical Reviews in Food Science and Nutrition, 51*:731–761.
- Linder C. & van't Riet Lievense (1994). Convective drying of bacteria, Factors influencing survival, *Advances in biochemical engineering and biotechnology, 51 (7),* pp.89.
- Lu S.-F., F.-L. Lee and H.-K. Chen (1999). A thermotolerant and high acetic acid-producing bacterium *Acetobacter* sp. I14–2, *Journal of Applied Microbiology, 86*, 55–62.

- Nguyễn Thế Trang (2010). Nghiên cứu sử dụng vi khuẩn lactic tạo chế phẩm bảo quản cá, *Viện khoa học và công nghệ Việt nam*. (In Vietnamese)
- Philipp Adler, Lasse Jannis Frey, Antje Berger, Christoph Josef Bolten, Carl Eric Hansen, Christoph Wittmann. (2014). The key to acetate: metabolic fluxes of acetic acid bacteria under cocoa pulp fermentation – simuating conditions, *Appl. Environ. Microbiol.* 4702- 4716
- Zahra Shokri, Mohamad Reza Fazeli, Mehdi Ardjmand, Seyyed Mohamad Mousavi and Kambiz Gilani. (20015) Factors affecting viabilty of *Bifidobacterium bifidum* spray drying. *DARU Journal of Pharmaceutical Science*
- Schwan, R.F. and Wheals, A.E. (2004). The microbiology of cocoa fermenta- tion and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition, 44:*205–221.
- Sandra Lagunes Gálvez; Gérard Loiseau; Jose LuisParedes; MichelBarel; Joseph-PierreGuiraud (2007). Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic, *Internatinal journal of food microbiology, Volume 111, Issue 1,* 124 – 130
- Strongmen, I.; Song, X. And Kramer, M. (1993) The application of heat pump in low temperature drying. *Heat pumps for energy efficiency of enviromental progress*, 341- 348.
- Vu T.L.A., Nguyen M.H., Phan T.H.(2017). Selection of Acetobacter species isolated from fermented cocoa beans in Dong Nai Province for their potential use as starter cultures. *Proceedings of the International Food Research Conference 2017*, Universiti Putra Malaysia, July 25-27 (2017): 452 - 455.

Studies on Traditional Making Process, Compositional Analysis and Microbial Population of Myanmar Traditional Tea Leaves, *Laphet*

Hnin Htet Htet Shein*, Kyaw Nyein Aye

Department of Chemical Engineering, Yangon Technological University, Yangon, Myanmar

*Corresponding Author (phiphilay2011@gmail.com)

ABSTRACT

The traditional fermented tea leaves, *Laphet*, is being consumed as a popular food in Myanmar over the centuries. It is consumed not only as a side dish, it is also as a main dish on the table. In some occasion, *Laphet* is being offered as a meaning of peace. As a fermented product of Tea Leaves, *Laphet* is being popular around the world in recent year. Tea leaves including young leaves and the leaf-buds of the tea plant are used for making Myanmar *Laphet*. It is done by natural fermentation process. As an initial study, the making process of *Laphet* is studied in the Southern Shan state of Myanmar. And then, the Compositional analysis such as; moisture - 70.10%, ash – 1.32%, crude fibre – 3.02%, crude protein – 5.53% and caffeine – 0.43%, and Microbial population such as; Total Plate Count (TPC), Yeast and Mould count (commercial and Lab-prepared) were examined by standard methods. As part of the main task, this work was done preliminary and from those result detailed of fermentation process can be further studied for the development of Myanmar Traditional *Laphet* processing.

KEYWORDS: Compositional Analysis, *Laphet*, Microbial Population, Tea, Traditional Fermentation Method

1 INTRODUCTION

Tea plant is well known plant all around the world and consumed widely because of its distinct taste and aroma. There are three major categories of tea: the non-fermented green tea which represents about 20% of world-wide tea consumption, the partially fermented oolong tea representing only 2%, and the fully fermented black tea with an 80% consumption.

In the tea preparation, tea leaves and flushes, which include a terminal bud and two young leaves, are plucked from *Camellia sinensis* bushes. There are five main processing steps in *Laphet* processing; (i) "*Laphet-chauk*" or dried tea leaf, (ii) "*A-Cho-Chauk*" or millk tea, (iii) "*Laphet-So*", (iv) "*Laphet-Si*" or tea leaf oil. *Laphet-chauk* or dried tea leaf, which is similar with the fully fermented black tea, is daily consumed as a water in almost all of the family in Myanmar. The second one *A-Cho-Chauk* as milk tea is mostly consumed as a breakfast drink. The third type; *Laphet-So* is the most popular for its unique taste and flavour. It is served as a greeting food and sometimes in history, it is offered as a peace food in war. The last type, *Laphet-Si* or tea leaf oil is used for natural hair dye for old age. *Laphet* or a traditional fermented tea leaves, is a lifestyle staple of Myanmar people since ancient time. Myanmar legend suggests that tea was first introduced into the country by King *Alaungsithu* of Bagan (1112-1167) who gave tea seeds to the people to cultivate. The people of the region accepted the tea seeds with one hand (La-ta-phat in Burmese language) and from there the term La-ta-phat, or *Laphet*, was used for tea in Myanmar. Tea leaf is also called as "Natt-thit-ywat'. In Myanmar fermented preparation, there are five main processing steps in tea processing; (1) withering, (2) rolling, (3) fermentation, (4) drying, (5) sorting and grading. Table 1 shows the composition of fresh tea leaves (Harler, 1964; Eden, 1965; Werkhoven 1988).

In Myanmar, tea plant is mainly cultivated in Shan State, the highland area. There are two traditional methods for making *Laphet* depends on the location, Southern Shan state and Northern Shan State. The main difference between the Northern and Southern Shan State is the heating method for withering. In the Northern Shan State, steaming method (for about 5 mins) is using and in the Southern Shan State, boiling (for about 2-3 mins) method is used.

The following processes; rolling, pressing, natural fermentation, and second-time rolling are

almost the same. Sometimes, the natural fermentation is processed in the underground temperature for obtaining constant temperature. Nowadays, in some areas, natural fermentation is only processed at the room temperature. Pindaya Township, Southern Shan state is taken as sample, making for home prepared *Laphet* to study the Composition, microbial population and the antimicrobial activity.

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Ericales
Family	: Theaceae
Genus	: Camellia
Species	: *Camellia sinensis*
Common Name	: Tea

Laphet processing is a fully fermented type by the natural flora. As a literature review, in Thailand traditional food, there is a one kind of fermented tea leaves almost the same processing with *Laphet*, called "Miang", mainly in Northern-Thailand. In the study of microbiology, the isolation of one strain of *Lactobacillus fermentum*, six strains of *Lactobacillus pantheris*, five strains of *Lactobacillus pentosus*, and four strains of *Lactobacillus suebicus* from fermented tea leaves was observed (Okada *et al.*, 1986; Gharaei-Fathabad and Eslamifar, 2011).

Figure 1: Tea Leaves

The bitter taste of fresh tea leaves is reduced after the *Laphet* fermentation. Furthermore, rinsing with warm water before serving is partially removed the bitterness (Han and Aye, 2015). Sometimes, Myanmar *Laphet* is expressed as "Heaven-in-the-mouth". The present work intends to study the compositional analysis, the microbial population and antimicrobial activity in home-prepared Myanmar *Laphet*. This work aims to promote the Myanmar traditional *Laphet* making process and to develop the Myanmar *Laphet* product.

Table 1: Composition of Fresh Tea Leaves (Werkhoven, 198)

Component	Dry Weight (%)
Insoluble in water	
Crude Fibre, Cellulose, Lignin, etc.	22
Protein	16
Fats	8
Chlorophyll and pigments	1.5
Pectin	4
Starches	0.5
Soluble in water	
Fermentable polyphenol	20
Other polyphenol	10
Caffeine	4
Sugars and Gummy matter	3
Amino acids	7
Minerals (Ash)	4

2 MATERIALS AND METHODS

2.1 Collection and Selection of Raw Materials

For the market sample, it is collected from the retailed shop. And for the lab-prepared sample, fresh young tea leaves containing leaf bud (from Southern Shan State, Pindaya Township) were collected and selected the newly harvested quality for the processing of tea to make *Laphet*.

2.2 Processing of Tea Leaves for Lab-prepared sample

The selected tea leaves are washed about two time to remove the dust. As the traditional method, the selected tea leaves were boiled about 5 minutes. The colour of fresh green tea leaves were changes from green to pale-green. And then the boiled leaves are spread on the mat for a while to cool down the temperature. Figure 2 shows the processing of Myanmar Traditional *Laphet*.

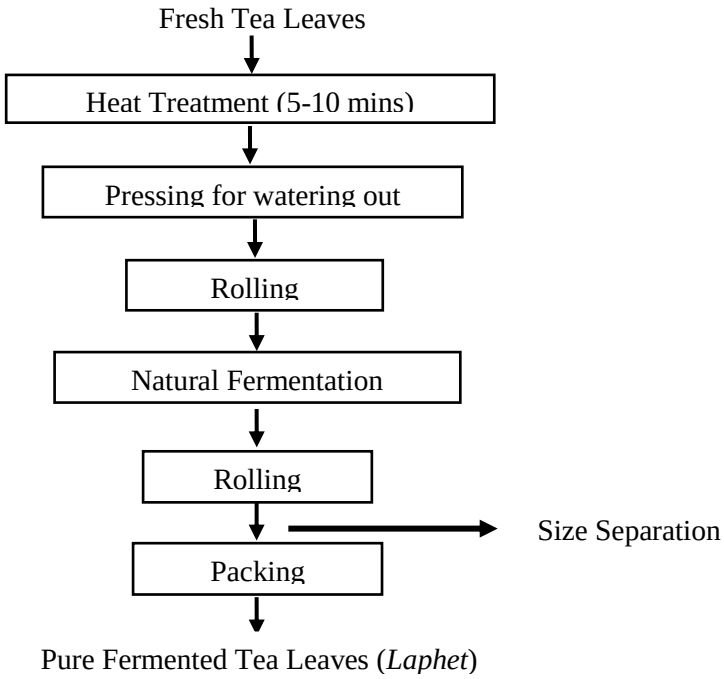


Figure 2: Myanmar *Laphet* Processing

And then, the leaves were rolled to twist by hand and by the machine for large scale production. The small amount of remaining water are coming out in this stage and squeezed out.

After all, the leaves are packed and pressed in the plastic bag not to have a space between layers. And then the plastics bag were placed in the upside down direction to come out the remaining water along the natural fermentation. The fermentation is processed at the controlled temperature, 37ºC to activate the natural micro-flora. After one month fermentation, the *Laphet* is used to study the following test.

In the large scale production, size separation is processed to select the high, middle and low range for sale market.

2.3 Compositional Analysis

To study the composition of fermented tea leaves (*Laphet*) the following parameters; Moisture, Crude Protein, Crude Fibre, Ash, and Caffeine (Micro Bailey - Andrew Method) are analysed. The analyses were done by the standard AOAC methods. This work was done at the "Food Industrials Development Supporting Laboratory (FIDSL)". The results are shown in Table 2.

2.4 **Microbial Population**

To study the microbial population, the two samples are tested the Total Plate Count, Yeast and Mold Count by Standard Bacterial Count Method (Online manual: USDA, Standard Opreating Procedure/Policy), and *E.coli* count by 3M petrifilm method. For the addition, Coliform count by MPN method, is tested the lab-prepared samples. This work was done at the "ALARM Ecological Laboratory, Biochemistry Laboratory". The results are shown in Table 3 and 4. (Sutton, 2010; USDA, 2015).

3 RESULT AND DISSUSIONS

To know the differences of composition between fresh green tea leaves and fermented tea leaves, some parameters are tested such as moisture, crude protein, crude fibre, ash and caffeine. The moisture percentage of fresh tea leaf (Whole shoot) is about 79%. As a result, after fermentation, it is reduced to 70.10%. And also the other parameters are also decreased when compared with fresh tea leaves.

By the literature review, the tea leaves is originally contain the Lactic Acid Bacteria, so that the lab-prepared samples is fermented at the 37 ºC to activate the natural flora; Lactic Acid Bacteria. By comparing the two samples, the lab-prepared samples is distinctly increased in the microbial count and the *E. coli* count is obtained the negative result. It can be assumed that the original microbes are well-growth at the controlled temperature. By comparing the result, the market sample would be contaminated the *E.coli* at the handling of end-product *Laphet* and the transportation from wholesale to retail transportation because of the poor packaging. The results are shown in table 3 and 4.

Table 2: Compositional Analysis of *Laphet* Sample

Sr. No	Test Parameter	Result (% Dry Weight)
1.	Moisture	70.10%
2.	Crude Protein	5.53%
3.	Crude Fibre	3.02%
4.	Ash	1.32%
5.	Caffeine	0.43%

#Analysed at the FIDSL Laboratory

Table 3: Microbial Population of Market *Laphet* Sample

Sr. No	The Parameter	Result
1.	Total Plate Count	$1.4x10^4$ cfu per g
2.	*Escherichia coli*	< 10 cfu per g
3.	Yeast and mould	$2.1x\ 10^5$cfu per g

#Analysed at the ALARM Ecological Laboratory

Table 4: Microbial Population of Lab-prepared *Laphet* Sample

Sr. No	The Parameter	Result
1.	Total Plate Count	$7.05x10^5$ cfu per g
2.	*Escherichia coli*	Negative
3.	Yeast and mould	$6.2x\ 10^5$cfu per g
4.	Coliform count	$>1.1x10^3$ MPN Index per 100 ml

#Analysed at the ALARM Ecological Laboratory

4 CONCLUSION

As the initial study, the difference between the fresh tea leaves and *Laphet* samples of compositional analysis is compared. In the microbial study, the Total Plate Count, Yeast and Mould Count and *E. coli* count are tested. As the *Laphet* is the fermented food product, the hygienic condition in end-product handling is important not to contaminate with foreign physical and chemical contaminants.

With a view of production on a larger scale of *Laphet*, it is essential to have a scientific understanding of the fermentation process. Even the Myanmar *Laphet* is popular food, the scientific study and quality control on the production is not enough. This can be developed by the study on *Laphet* Fermentation with the selected microbe to reduce fermentation time and to standardize and control the processing steps and the end-product development as further study.

5 ACKNOWLEDGEMENT

The author would like to express the grateful to all the teachers and colleagues for their accomplished guidance, willingness to share ideas and suggestions for completing this paper. Special thanks dedicated to my beloved parents for supporting throughout the study by mentally and physically.

REFERENCES

- Eden, T. (1965). TEA*: The Development of Tea Culture*, (Second Edition).
- Gharaei-Fathabad, E., & Eslamifar, M. (2011). Isolation and applications of one strain of Lactobacillus paraplantarum from tea leaves (Camellia sinensis). *Am. J. Food Technol*, *6*(5), 429-434.
- Han, T., & Aye, K. N. (2015). The legend of laphet: a Myanmar fermented tea leaf. *Journal of Ethnic Foods*, *2*(4), 173-178.
- Harler, C. R., (1964). The Culture and marketing of Tea: The Tea Plant.
- Manual of 3M Food Safety, *E. coli*/ Coliform count plate, Available online: www.3M.com/Food-Safety
- OKADA, S., DAENGSUBHA, W., Uchimura, T. A. I., OHARA, N., & KOZAKI, M. (1986). Flora of lactic acid bacteria in Miang produced in northern Thailand. *The Journal of General and Applied Microbiology*, *32*(1), 57-65.
- Sutton, S. (2010). The most probable number method and its uses in enumeration, qualification, and validation. *Journal of Validation Technology*, *16*(3), 35-38.
- USDA (2015). Centre of Veterinary Biologics, Standard Opreating Policy/Procedure, Standard Bacterial Plate Count, BBSOP0019.04, (February 27, 2015)
- Werkhoven, J., (1988). Tea Processing, Food and Agriculture Organization (FAO, United Nation),

Studies on Doxycycline Degradation Fraction in Milk Curd and Whey by Plant Rennet

Aye Moe Thwe*, Swe Swe Win

Department of Chemical Engineering, Yangon Technological University, Yangon, Myanmar
*Corresponding Author (ayemoe.thwelay@gmail.com)

ABSTRACT

Doxycycline, belonging to the Tetracyclines is a broad-spectrum antimicrobial agent which is widely used in preventing and treating infectious diseases in the farm of food-producing animals. This extensive use can lead to an increase in their residues levels in food of animal origins. The main purpose of this work is to study the degradation of doxycycline during the production process of milk curd and whey, using plant rennet. In this paper, doxycycline spiked milk was processed with traditional method to produce curd using *C.gigantea* (Ma-yo-gyi) leaves and latex. The percent degradation was analyzed using high performance liquid chromatography (HPLC) with ultraviolet detection and gradient elution. According to the above analysis, the percent reduction due to proteolytic enzymes in different plant sources was found to be about 40-60%. The degradation fraction due to leaves and latex in whey was about 11-13% and curd was about 20-40%. Results in the present study showed that the fraction in the curd was more than in whey due to protein binding capacity of doxycycline and the casein hydrolysis activity of proteolytic enzyme in leaves and latex. The latex has higher milk coagulation activity than leaves. The heat and enzymatic activity during curdling is one of the technical aspect of doxycycline degradation in residues reduction for safer food consumption.

KEYWORDS: *C.gigantea*, Doxycycline, HPLC, Milk Curd, Whey

1 INTRODUCTION

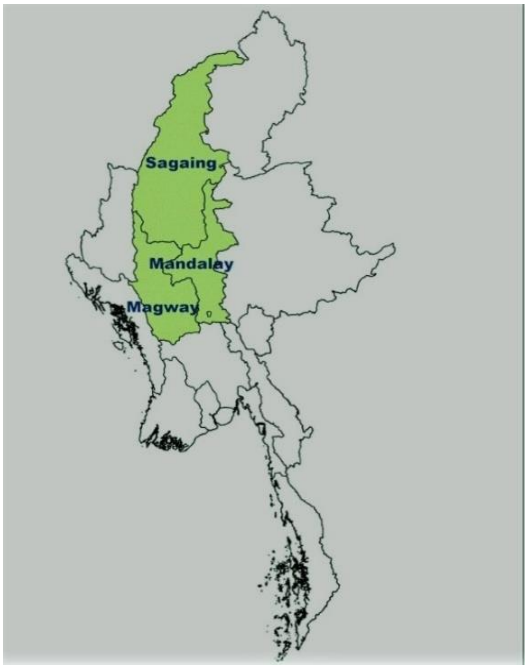
The indigenous plant *Calotropis C.gigantea* R.Br., is well known by its vernacular name Ma-yo-gyi in Myanmar and common name is giant milk-weed. The plant belongs to the family Asclepiadaceae. This plant is widely distributed in Myanmar as wild plant, especially in dry zones of Mandalay, Sagaing and Magway Division. It was formely introduced into India, Africa and Malay Peninsula (Khine, 1998).

This plant is a perennial shrub, about 2m high, latex present, stem cylindrical, thick, young parts densely floccose. Leaves are opposite, simple, petioles are very short to almost sessile, 7.5-15.0 cm long, 5.0-7.5 cm in diameter. The flowers which are medium size, umbeliform cymes. The colours of flowers are variable, purple or pink or white and the fruits with 8-10 cm long, recurved, smooth. But the fruits and seeds are rarely found. It is found growing in open places, flowering and fruiting from January to May. This plant grows readily in warm, humid climates, performing best in full sunlight (Aye, 1993).

Presence of latex is one of the characteristic features of plants belonging to the families Euphorbiaceae, Asclepiadacae, Moraceae and Apocyanaceae. Enzymes from plant lattices are of widespread interest due to their involvement in various physiological functions and economic benefits. Presence of multiple proteases in plant lattices in abundant quantity makes it a potential plant coagulant source *C. gigantea*, belonging to Asclepiadacae, have been used in folk medicine for various ailments (Anusha *et al*., 2014).

The plant is purgative, anthelmintic, alexipharmic, cures leprosy, leucoderma, ulcers, tumors, piles, diseases of the spleen, the liver, and the abdomen; the juice is anthelmintic and leucoderma, tumors, ascites, diseases of abdomen. The leaves are applied to paralyzed parts, painful joints, swellings; heal wounds. The tincture from the leaves used as antiperiodic in cases of intermittent fevers, inflammations, tumors, rat-bite, good in ascites. The milk is bitter, heating, purgative; Laxative; cures piles. The root bark is diaphoretic; cures asthma and syphilis. Flower is sweet, bitter, anthelmintic, analgesic, astringent, cures piles *C. gigantea* latex contains cysteine proteases similar to papain such as

calotropain FI, FII, calotropin DI and traces of glutathione. Leaves contain alkaloids, glycosides and mudarine (Singh *et al.,* 2014).



A B

Figure 1: Localization of the *Calotropis gigantea* origin A-Geographic mainly plant distribution area B-*Calotropis gigantea* plant

The leaves, latex, stem and flowers of *C.gigantea* have different milk clotting activity and leaves and latex were widely used in curdling of milk. Latex also has higher milk clotting activity when compare to other parts. The latex and leaves were traditionally used plant milk coagulants and used in preparation of the curd which was eaten by frying or as a curry cooked with tomato in Myanmar. Protease in *C.gigantea* was closely similar to rennin in casein hydrolysis pattern and hence could serve as a promising vegetable coagulant. Milk clotting activity was maximum at 37 °C and declined with further increased in temperature whereas *C.procera* show optimal proteolytic and milk clotting activity at 60 °C and 70°C respectively. All plant part has optimum milk clotting activity at pH 5.5. This enzyme predominantly cleaves Phe105-Met106 bond in κ-casein, depilating the casein micelle and inducing the milk clotting (Anusha *et al.,* 2014).

Doxycycline, belonging to the Tetracyclines is a broad-spectrum antimicrobial agent which is widely used in preventing and treating infectious diseases in the farm of food-producing animals. However, they have a range of side effects, including disturbances in healthy intestinal microflora, allergic reactions, liver and kidney malfunctions, hypersensitiveness and intense-light related dermatitis and the extensive use can lead to an increase in their residues levels in food of animal origins and is not for used in lactating cattle and layers. Moreover, nowadays it is necessary to take into account the relatively high probability of acquired tetracycline resistance. In order to protect consumers' health, EU legislation lays down the maximum residue limit (MRL) in food of animal origin for veterinary medical products approved for use in food producing animals.

Table 1: MRL of Doxycycline in difference species and target tissues (European Medicine Agency, 2015)

Pharmacologically active substance	Marker Residue	Animal Species	MRLs	Target tissue	Other Provision
Doxycycline	Doxycycline	Bovine	100 µg/kg 300 µg/kg 600 µg/kg	Muscle Liver Kidney	Not for use in animals from which milk is produced for human consumption
		Porcine	100 µg/kg 300 µg/kg 300 µg/kg 600 µg/kg	Muscle Skin+ fat Liver Kidney	
		Poultry	100 µg/kg 300 µg/kg 300 µg/kg 600 µg/kg	Muscle Skin+ fat Liver Kidney	Not for use in animals from which milk is produced for human consumption

The presence of veterinary drug residues in milk is important from the hygienic point of view as an important quality marker, and also from the technological point of view. Presence of drug residues poses a risk especially for dairy product manufacture, for it may interfere with dairy cultures (AOAC, 2000). The main objective of this paper was to determine the degradation fraction of Doxycycline in curd and whey by plant rennet using traditional method of curdling in Myanmar.

2 MATERIALS AND METHODS

2.1 Reagents

All the chemicals used were analytical grade. Doxycycline hyclate standard were purchased from AK Scientific (USA). Oxalic acid, dibasic sodium phosphate (Na_2PO4),Citric acid , EDTA, Sodium chloride which were analar grade from Veterinary Assay Lab.Solvents of Acetonitrile, methanol and water were HPLC grade (Advanced Diagnostic Company, Waters).McIIvaine-EDTA-NaCl buffer was prepared according to the method of AOAC (2000).

2.2 Apparatus

The apparatus includes an analytical balance, refrigerated centrifuge, hot plate, pH meter and vortex mixer, lactoscan, high performance liquid chromatography.

2.3 Milk Sample

Antibiotics free milk which was already screened with The AuroFlow TM BTS Combo Strip" test kit was ordered from Silver Pearl Dairy Farm. The sensitivity limits for Doxycycline of the test kit was 50-70ppb. Physicochemical properties of milk such as fat, salt, solid non- fat, protein, lipid, water content, density, temperature and freezing point were analyzed with lactoscan.

2.4 Curd Processing with Leaves and Latex

Curd production was carried out with traditional method. Two leaves of *C.gigantea* about 4 g was triturated in 500 ml of fresh raw milk. For the processing with latex, the latex from the stem about 0.06g was added to 500ml of fresh raw milk. The milk was then heated to 70 °C on water bath for 30 min. After the milk was fully coagulated, it was cooled in water for 10min and the whey-coagulum mixture was poured into a basket containing a cloth and sited in a test-tube to collect and to quantify the whey. The leaves were taken out from the coagulum. The setup was left for the whey to filter through

till it stopped dripping. The cloth with coagulum inside was folded, tied and let out the remaining whey, it was dried for approximately 3 hours to have a constant dry weight.

2.5 Sample Extraction

A 2.0 ml of McIIvaine-EDTA-NaCl buffer was added to 2ml of test milk, whey and 2g of curd sample. The curd was ground in motor with the pestle. The mixture was mixed on a vortex mixer for 10 min, centrifuged at 5000 rpm for 30 min at 15 °C. The supernatant was collected, diluted with water and then filtered through 0.2µm syringe filter into vials for HPLC analysis (Tian *et al.*, 2017).

2.6 HPLC Analysis

10 µL sample was injected into HPLC system, (Waters e2695, USA) equipped with UV/Vis detector 2489, auto sampler. The system was controlled and the data was analyzed using Empower software. A chromatography column C18, 100 x 4.6mm with the particle size of 2.7 µm was used. UV detection was carried out at the wavelength of 355nm.The measurement was taken place in gradient mode. The mobile phase A was 10mM oxalic acid in water and mobile phase B was 10mM oxalic acid in acetonitrile. The mobile phase flow rate of (0.5ml/min) was used in a gradient mode of 15% B initial, linear gradient to 50% B in 8.00 min, held until 11.25min, back to 15% B at 11.60 min, hold and equilibrate until 12.85 min. The sample injection volume was 10µl and the column temperature was 30˚C. Each sample of whey and curd of milk were analyzed in duplicates, every series containing a blank sample to confirm the milk was antibiotics free (Young and Tran, 2013).

3 RESULTS AND DISCUSSIONS

The present study showed that the doxycycline degradation fraction in curd due to heat and enzymatic activity was more than in whey. The casein hydrolytic activity by the protease enzyme in *Calotropis gigantea* during milk curdling reduces doxycycline about 40-60%.The degradation percent was higher than boiling of milk which contains only heat treatment. The degradation fraction in the curd was higher than the whey due to protein binding capacity of doxycycline which is 90%. The present work focused on the degradation fraction in curd and whey by plant rennet from different plant sources like from leaves and latex. The percent reduction in curd and whey by plant rennet during curdling is described in table 2.

Table 2: Percent degradation of Doxycycline by plant rennet (Leaves, Latex)

Plant Rennet	Added amount	Found amount	Curd	Whey	% Reduction
Latex	500mg	466.75mg	201.5 mg	54.94mg	45.06%
	550mg	517.69mg	211.9 mg	67.38mg	47%
Leaves	550mg	517.69mg	171.44mg	68.67mg	53.62%
	700mg	676.26mg	179.13mg	86.54mg	60.72%

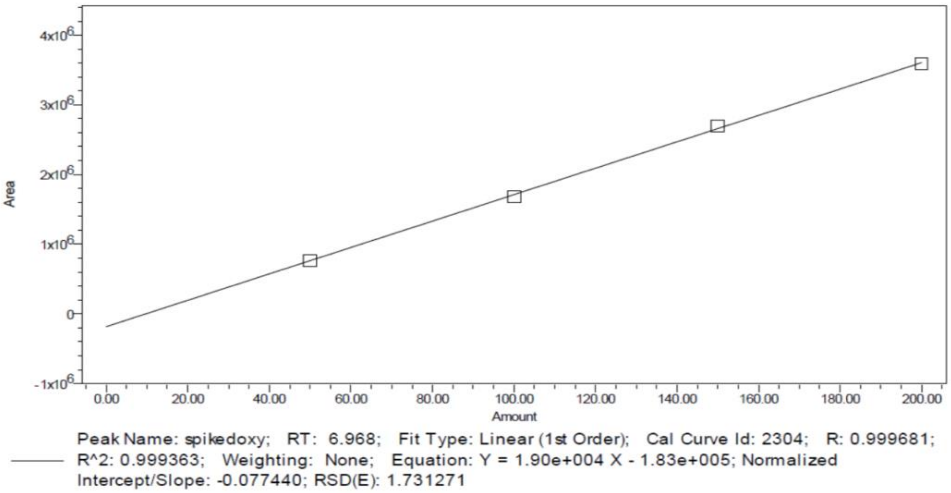
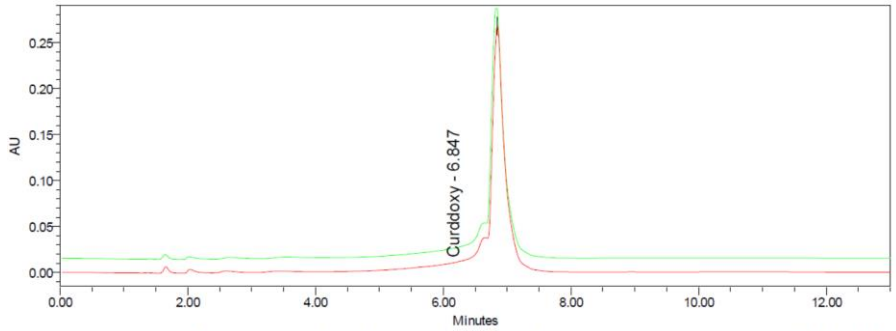
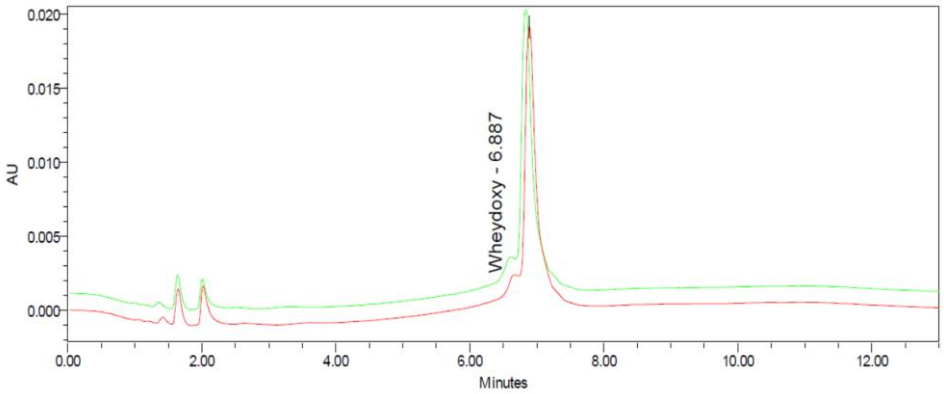


Figure 2: Standard Calibration Curve for Doxycyline



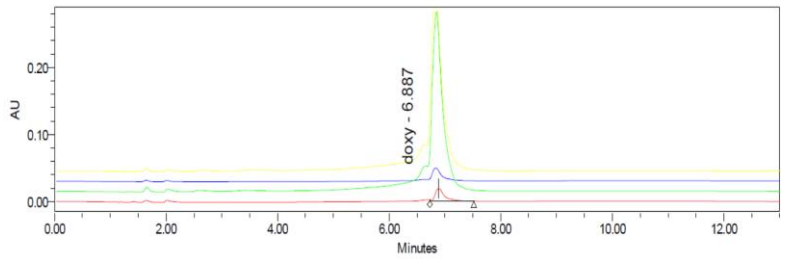
Sample Name	Vial	Inj	Channel	RT	Area	Height	Amount	Conc:	Unit
curdleafdx	3	1	W2489 ChA	6.847	3588062	259845	199.006	19.901	ppm
curdlatdx	5	1	W2489 ChA	6.833	3704736	267661	205.163	20.516	ppm

Figure 3 Chromatogram of Doxycycline degradation fraction in curd by plant rennet (leaves,latex)



Sample Name	Vial	Inj	Channel	RT	Area	Height	Amount	Conc:	Unit
wheyleafdx	2	1	W2489 ChA	6.887	223243	18554	21.460	2.146	ppm
wheylatdx	4	1	W2489 ChA	6.837	245092	18881	22.612	2.261	ppm

Figure 4: Chromatogram of Doxycycline degradation fraction in whey by plant rennet (leaves,latex)



Sample Name	Vial	Inj	Channel	Name	RT	Area	Height	Amount	Conc:	Units
wheyleafdx	2	1	W2489 ChA	spikedoxy	6.887	223243	18554	21.460	2.146	ppm
curdleafdx	3	1	W2489 ChA	spikedoxy	6.847	3588062	259845	199.006	19.901	ppm
wheylatdx	4	1	W2489 ChA	spikedoxy	6.837	245092	18881	22.612	2.261	ppm
curdlatdx	5	1	W2489 ChA	spikedoxy	6.833	3704736	267661	205.163	20.516	ppm

Figure 5: Chromatogram of Doxycycline degradation fraction in curd and whey by plant rennet (leaves,latex)

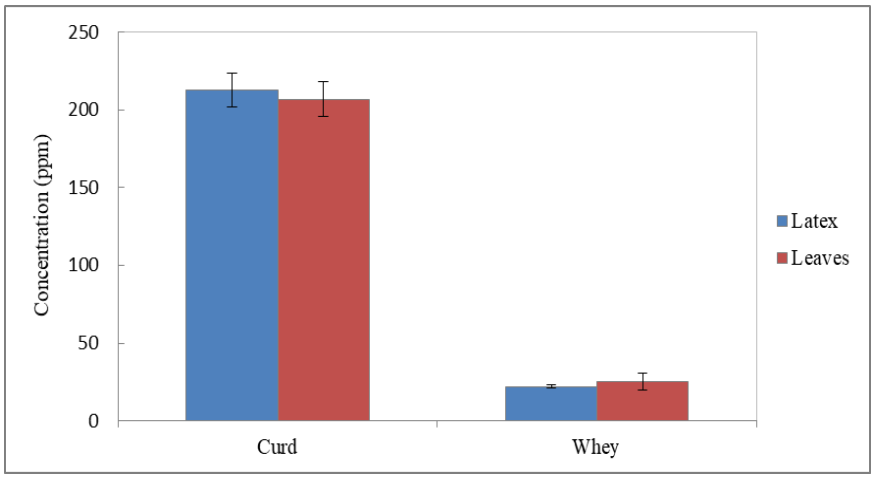


Figure 6: Doxycycline fraction in curd and whey by plant rennet

According to the analysis, percent reduction of doxycycline by latex and leaves was about 46.03% and 57.17%. The fraction of doxycycline by latex in whey was about 12.39% (21.953ppm) and curd was about 41.58% (216.131 ppm). The yield of whey and curd was about 139ml and 48.68g in 250 ml of milk. The fraction in whey and curd by leaves was about 13.02% (25.154ppm) and 29.8% (206.933ppm) and the yield was about 155ml of whey and 42.38 g of curd in 250 ml of milk.

Comparing the results of curdling by leaves and latex, the higher yield of curd, solid mass of milk protein, was found in latex curd than leaves curd because of the latex has stronger protease activity and higher milk clotting activity compare to the leaves. So, more degradation fraction of doxycycline contain in curd was found due to the strong protein binding capacity of doxycycline. But in case of whey, the yield was higher in whey made by leaves as leaves contain lower protease enzyme and milk clotting activity. The percent recovery of Doxycycline was higher in curdling by latex and the percent reduction was higher in curdling by leaves because more time is required for curdling as leaves contain less protease enzyme. The yield could be influenced by a number of factors like water, fat, protein, lipid and mineral content of milk.

4 CONCLUSION

According to the present findings, the more Doxycycline degradation fraction was found in curd made by latex. The percent reduction due to proteolytic enzymes in different plant sources was found to be about 40-60%. The degradation fraction due to leaves and latex in whey was about 11-13% and

curd was about 40-60%. Different plant sources have different proteolytic activity and the latex has higher milk coagulation activity than leaves. From technological point of view, the traditional method of use of vegetable coagulant in curdling was one of the technical aspect of doxycycline degradation in residues reduction for safer food consumption. Moreover, this paper also intended to determine the maximum residues limit of antibiotics in this country.

Thermal processing may not only reduce the concentration of drug residues in food, but it can also change their pharmacological and toxic effects. Heat treatment may give rise to new chemical compounds with higher levels of toxicity than that of the parent compound. The presence of residues at much higher level in foods may constitute a variety of public health hazards including toxicological, microbiological, immunological and pharmacological hazards. Most important health aspects which should be taken into account are: possible impact on the emergence of antimicrobial resistance for antimicrobials administered in human therapy. Further studies are needed to determine the metabolite in antibiotic treated cow's milk from safety point of view.

5 ACKNOWLEDGEMENT

The author especially appreciates and thanks to all her teachers and all people who help directly or indirectly towards the completion of this paper. Special dedication and deep appreciation is extended to my beloved father for his valuable encouragement and support throughout the study.

REFERENCES

- Anusha, R., Singh, M. K., & Bindhu, O. S. (2014). Characterisation of potential milk coagulants from Calotropis gigantea plant parts and their hydrolytic pattern of bovine casein. *European Food Research and Technology*, *238*(6), 997-1006.
- AOAC (2000). William Horwitz, Official Methods of Analysis of AOAC Internationals (17th Edition), Food Composition, Additives; Natural Contaminants, Volume II, *Chapter 33*, Dairy Products.
- Aye, Y.Y. (1993). "*Kinetic Studies of Asclepain from Latex of Milk Weed* (*Asclepias gigantea*)", University of Yangon.
- European Medicines Agency (2015). *European public MRL assessment report (EPMAR), Doxycycline (all food producing species*, Commission Regulation (EU) No 37/2010. http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2015/02/WC500183123.pdf
- Fedeniuk, R. W., & Shand, P. J. (1998). Theory and methodology of antibiotic extraction from biomatrices. *Journal of Chromatography A*, *812*(1-2), 3-15.
- Kellnerová, E., Navrátilová, P., & Borkovcová, I. (2015). Effect of pasteurization on the residues of tetracyclines in milk. *Acta Veterinaria Brno*, *83*(10), 21-26.
- Khine, H. T. T., (1998) "*A Pharmacognostical Study of Calotropis gigantea R.Br*", University of Yangon, Department of Botany.
- Singh, N., Gupta, P., Patel, A. V., & Pathak, A. K. (2014). Calotropis gigantea: A review on its Phytochemical & Pharmacological Profile. *International Journal Parmocology*, *1*(1), 1-8.
- Tian, L., Khalil, S., & Bayen, S. (2017). Effect of thermal treatments on the degradation of antibiotic residues in food. *Critical reviews in food science and nutrition*, *57*(17), 3760-3770.
- Wang, J., MacNeil, J. D., & Kay, J. F. (Eds.). (2011). *Chemical analysis of antibiotic residues in food* (Vol. 38). John Wiley & Sons.
- Young, M. S., and Tran, K. V. (2013). HPLC/UV determination of Tetracyclines in Milk using Mixed-Mode SPE and eXtended Performance [XP] 2.5µm Columns, Application note, Waters cooperation Milford MA, USA.

Determination of Total Fat and Iodine Value in Glutinous Rice-Based Oily Snacks (*Htoe Mont*)

Aye Thinzar Khine*, Myint Myint

Department of Chemical Engineering, Yangon Technological University, Yangon, Myanmar
*Corresponding Author (zindmontun1992@gmail.com)

ABSTRACT

Most of Myanmar traditional foods are based on rice and glutinous rice. One of the popular traditional foods is *Htoe Mont* which is the product of Mandalay region (Upper Myanmar). This study aim to determine the total fat content of Fried Coconut *Htoe Mont* which was made of glutinous rice, sugar, peanut oil, peanut and coconut; its results were shown various percentages between 6% and 16.9% of total fat content and Fruit *Htoe Mont* which was made of glutinous rice, sugar, peanut oil, peanut, coconut, raisin and cashew nut were included; its total fat contents were shown as 2.5%-11.7%. The results of plain *Htoe Mont* (after removed ingredients from *Htoe Mont*) were shown that constant result was approximately 2.00 %. This study was done on fat extract in these samples which are determined by Soxhlet Extraction Method.

Then, the iodine value (IV) of oils in *Htoe Mont* which were collected five types of brand was determined. Determination of iodine value was analyzed by Wijs Method. This study showed that the total fat content of *Htoe Mont* depend on different ingredients and weight of ingredients, oil in various brands of *Htoe Mont* were peanut oil as results.

KEYWORDS: Fats, Snacks, *Htoe Mont*, Iodine Value, Soxhlet, Wijs Method

1 INTRODUCTION

In modern living, the popularity of snacks was attributed to more leisure time. Snack defines as simple meal that is quick to cook and to eat (Win, 1999). Moreover, traditional foods are foods that were consumed before the modernization and industrialization of the food supply. Traditional food is an important part of the culture. In Myanmar, traditional snacks are prepared from various raw materials. Generally, traditional snack producers are lack of knowledge and manufacturing their products without consulting food technicians. Especially, most of the snacks are prepared for favourable attention on the market. Eating too much of snacks, consequently, is bad for human health (Campbell, 2018).

There are various types of snacks in local market. They are-

(a) Variety of pickles (mainly based on fruits and vegetables)
(b) Milk and dairy products
(c) Egg based snacks
(d) Cereal and grain based snacks (especially wheat, rice and glutinous rice based products)
(e) Sweet snacks
(f) Peas and Beans based snacks (Chit, 1976).

It is true without a doubt that the many kinds of Myanmar traditional snacks are fundamentally prepared with sticky rice or glutinous rice, ordinary rice and both sticky or glutinous and ordinary rice. And also Myanmar traditional snacks contain other ingredients like jaggery, oil, sesame seeds, coconut, sugar, groundnut and butter, etc. (Thwe, 2015).

Myanmar, many kinds of dessert is inspired by Chinese, Indian and Thailand due to frontier of a state or culture. Among many kinds of traditional foods, *Htoe Mont* is considered as one of the popular national snacks of Myanmar which is based on glutinous rice containing other ingredients such as sugar, peanut, oil, sesame seeds, coconut pieces, resin, cashew nut, butter, milk cream, etc. (Wikipedia contributors, 2018).

There are various brands of glutinous rice oily snacks in Mandalay region. Most of the Myanmar traditional snack factories are found near the *Eain Taw Yar* Pagoda in cultural city. Especially, Mandalay *Zay Cho* is the biggest wholesale market in upper Myanmar and another place is near the Mandalay Railway Station. Both are the famous places of selling for traditional snacks.

U Kaukya and *U Gyi* are formely famous brands of *Htoe Mont* in Mandalay. Nowadays, *Myint Myint Khin* and *Tin Tin Aye* are recently famous brands of glutinous rice-based oily snacks.

In Thailand, *Khanom Baa Bin* (Fried Cake) is the similarity of *Htoe Mont.* And also *Nian gao* (Chinese New Year's cake, *Tikoy*) is one of the sticky sweet snacks which is similarity of Myanmar traditional snacks, *Htoe Mont* (Turton, 2016). The popular Myanmar traditional snacks, different types of *Htoe Mont*, are shown in Figure 1.

Figure 8: Glutinous Rice Oily Snacks (Htoe Mont)

2 MATERIALS AND METHODS

2.1 Sample Collection

Collection and selection of fresh Myanmar *Htoe Mont* samples with 10 days shelflife of various brands were carried out in Mandalay.

2.2 Preparation of Sample for Analysis

All samples for total fat content were prepared and tested according to Official Methods of Analytical Chemists (AOAC 1980).

Furthermore, to determine the Iodine Value (IV) of oils in different five brands of *Htoe Mont* were prepared and tested according to according to International Union of Pure and Applied Chemistry (IUPAC) (Wikipedia contributors, 2018).

2.3 Experimental Studies

The collected samples were determined as follows:

1. Total fat content in Fried Coconut *Htoe Mont*, Fruit *Htoe Mont* and plain *Htoe Mont* (after removing additional ingredients from *Htoe Mont*)
2. Iodine Value (IV) of oil in various brands of *Htoe Mont*.

2.3.1 Determination of Total Fat Content by Soxhlet Extraction Method

1-1.1 gm of each dry food sample was washed with petroleum ether and weighed into a filter paper thimble. The thimble containing the dried materials was placed in a Soxhlet apparatus and the extraction allowed proceeding for 5 hours. The solvent as petroleum ether (boiling point, 50-60°C) was placed in the flask which was heated by means of a water bath on a hot plate or by an electric bulb. The volatilized petroleum ether on reaching condenser condensed and dropped on the thimble and covered

it. When completely covered, the petroleum ether siphoned off and returned to the flask and the process was repeated.

After the extraction was completed (usually it takes about 5 hours with most foods), the solvent was distilled off, and the flask was dried at 100°C or in an incubator to constant weight and weighed. The weight difference gives the amount of fat in the weight of food sample taken (AOAC, 1980).

2.3.2 Determination of Iodine Value (IV) by Wijs Methods

0.2 g of sample was placed into 250 ml iodine flask. And carbon tetrachloride, CCl_4, 20 ml was added to dissolve the sample and 25 ml of Wijs solution, (iodine monochloride) was measured with a pipette and added into the iodine flask. Besides, the flask was securely closed with the stopper vessels and occasionally shacked and allowed to stand at 25± 5°C for 30 min without being exposed to light. Then, 20 ml of 15% potassium iodide (KI) and 60 ml of carbon dioxide free water were added. Finally, the prepared sample was titrated with 0.1 N sodium thiosulphate, $Na_2S_2O_3$. When the titrated colour became quite pale, 0.5 ml of starch indicator was used and the end point indicated as colourless.

The method to be used for specific named vegetable oils is stipulated in the standard [10].

3 RESULTS AND DISCUSSION

In this research, the results of total fat content of traditional glutinous-based oily snacks analysis were presented in Table 1, 2 and 3.

In Table 1, the results of total fat content of Fried Coconut *Htoe Mont* are 16.9%, 7.6% and 6.3% respectively. There is a significant difference in the proximate composition of the samples because of the ingredients such as glutinous rice, sugar, peanut oil, peanut and fried coconut. Sample preparation was important because the samples were not homogenized. Consequently, the snack samples were made based on glutinous rice and they were difficult to homogenize. So, the different ingredients which are of different amounts must be included in the different prepared samples. The detailed results are shown in Table 1.

Table 1: Determination of total fat content in Fried Coconut *Htoe Mont*

Brand Name	Code No	Sample No.	Sample Weight (g)	Cup Weight (g)	Cup Weight+ Fat Weight (g)	Fat Content $^{w}/_{w}$	Total Fat Content Results %	Total Fat Content (Average Results)
A1	ATZK -301	I	1.1680	107.9626	108.1603	0.16	16.9	Do not determine due to huge variation
		II	1.1664	107.7615	107.8511	0.07	7.6	
		III	1.1610	107.8690	107.9425	0.06	6.3	

Analyzed at Food Chemical Laboratory in Nay Pyi Taw

Secondly, the results of total fat content of Fruit *Htoe Mont* are shown in Table 2.They are 11.7%, 3.7% and 2.5%.Also, there is a significant difference in the proximate composition of the samples. In Fruit *Htoe Mont*, glutinous rice, sugar, peanut oil, peanut, coconut pieces, raisin and cashew nut were included. Therefore, the results are similar to the former phenomena. The detailed results are shown in Table 2.

Table 2: Determination of total fat content in Fruit *Htoe Mont*

Brand Name	Code No	Sample No.	Sample Weight (g)	Cup Weight (g)	Cup Weight+ Fat Weight (g)	Fat Content $^{w}/_{w}$	Total Fat Content Results %	Total Fat Content (Average Results)
A2	ATZK -302	I	1.1600	111.4875	111.6232	0.11	11.7	Do not determine due to huge variation
		II	1.1621	109.8799	109.9247	0.04	3.7	
		III	1.1677	112.4444	112.4983	0.02	2.5	

Analyzed at Food Chemical Laboratory in Nay Pyi Taw

Subsequent analysis results are shown in Table 3. This table indicates the results of plain *Htoe Mont* (after removing additional ingredients from *Htoe Mont*). The average results of plain *Htoe Mont* code number ATZK-301 and ATZK-302 are found to be 2.07% and 2.03% respectively. Removing the additional ingredients from the samples leads to the constant results.

Table 3: Determination of total fat content in plain *Htoe Mont* (after removing additional ingredients from *Htoe Mont)*

Brand Name	Code No.	Sample No.	Sample Weight (g)	Cup Weight (g)	Cup Weight+ Fat Weight (g)	Fat Content $^{w}/_{w}$	Total Fat Content Results %	Total Fat Content (Average Results)
A1	ATZK -301	I	1.0248	107.9580	107.9833	0.02	2.5	2.07%
		II	1.0104	107.8601	107.8791	0.02	2.0	
		III	1.0462	116.5398	116.5574	0.02	1.7	
	ATZK -302	I	1.0650	107.4594	107.4784	0.02	1.8	2.03%
		II	1.0698	107.8689	107.8949	0.02	2.4	
		III	1.0658	107.8673	107.8873	0.02	1.9	

Analyzed at Food Chemical Laboratory in Nay Pyi Taw

Finally, the iodine value (IV) of oils in *Htoe Mont* sample of five brands which were randomly collected from different townships was determined. All the tested samples' results are considered peanut oil and according to Codex Alimentarius Commission the range of peanut oil value is shown in Table 4.

Table 4: Determination of Iodine Value (IV) in various brands of *Htoe Mont*

No.	Brands of *Htoe Mont*	Iodine Value (IV)	
		From Experiment Results	Reference value of peanut oil from Codex Alimentarius Commission
1	A1	95	86-107
2	B1	89	
3	C1	87	
4	D1	87	
5	E1	87	

Analyzed at Laboratory of Food and Drug Administration, Mandalay Branch

4 CONCLUSION

In this research work, experimental studies have shown that the results of total fat content of *Htoe Mont* samples depend on the different ingredients and different amounts of ingredients. Especially, most of the Myanmar traditional snacks are not supposed to have the constant quality even from the same batch of the products and production line. The fat from the various ingredients (i.e. peanut, cashew nuts and coconuts) prevails in the final products of fats.

Furthermore, the selected various brands of *Htoe Mont*, five samples used the peanut oil. According to the results, most of the Myanmar traditional oily snacks (*Htoe Mont)* in Mandalay (Upper Myanmar) markets use peanut oil.

Further studies should be conducted in such fields as the selection of raw materials, proper packaging, and good handling practice, healthy and expert workers in *Htoe Mont* (Myanmar popular snacks from Mandalay)

In a nutshell, the findings of this study are expected to contribute to the production of good quality Myanmar traditional snacks and towards the positive changes. However, it is advisable to perform the traceability of raw materials, process quality control, nutritional labeling and products labeling according to Codex guide line. Only then, Myanmar traditional snack industries will be developed and stand qualified in the future markets.

5 ACKNOWLEDGMENTS

The author would like to express her grateful thanks to all the teachers from Department of Chemical Engineering, Yangon Technological University and Department of Food and Drug Administration, Nay Pyi Taw and Mandalay Branch for their accomplished guidance, willingness to share ideas and suggestion for completing this paper.

REFERENCES

- AOAC Official Methods of Analysis, thirteen editions. (1980).Pearson's Chemical Analysis of Foods, eight editions. (1981).
- Campbell, M. (April 09, 2018). *Bad Effects of Snacking* from http://healthyeating. sfgate.com/ bad-effects-snacking-3832.html
- Chit, K. M. (1976). *Colourful Burma: Her Infinite Variety: a Collection of Stories and Essays*. Khin Myo Chit Theidi Sazin.
- Codex Committee on Fats and Oils (CCFO), (19-23 February 2001), London, according to IUPAC 2.205/1, *Wijs method*, or ISO 3961:1996 http://www.fao.org/docrep/meeting/005/X9919E/x9919e0e.htm
- Harold, E., Ronald, S. K., & Ronald, S. (1981). Pearson's chemical analysis of foods. *Churchill Livingstone, Edinburgh, UK*.
- Thwe, A.M., (June, 2015). *Comparative Studies of Myanmar Traditional Snacks*, D.F.T Term Paper, YTU, Myanmar.
- Turton, Natasha (August 18, 2016). *Kanom Babin (Coconut Cake).*
- Weins, M. (2018). Authentic Village Food and Life with the Khmu People in Laos. Available online at www.mingrationology.com
- Wikipedia contributors. (2018, August 4). Burmese cuisine. In *Wikipedia, The Free Encyclopedia*. Retrieved 04:49, September 01, 2018, from https://en.wikipedia.org/w/index.php?title=Burmese_cuisine&oldid=853333397
- Win, S.S., (March, 1999). *Product Development on Potato Based Snacks*, M.F.T Thesis, YTU, Myanmar.

Effect of Milling on Arsenic Contents in Long-Grain Rice

Yaowalak Rooppat[1], Wenika Benjapong[1*], Jintana Sirivararsenicai[2], Rodjana Chunhabundit[2], Weeraya Karnpanit[1], Sirirat Khundam[1], Jakkit Sakolkittinapakul[1]

[1]Institute of Nutrition, Mahidol University
[2]Graduate Program in Nutrition, Faculty of Medicine, Ramathibodi Hospital, Mahidol University
*Corresponding Author (wenika.ben@mahidol.ac.th)

ABSTRACT

Arsenic is a toxic metal that is naturally distributed in soil and water. Rice can absorb arsenic much more effectively than other grains. Brown rice usually contains arsenic at higher level than polished rice. This study aimed to determine the effect of milling on arsenic contents in long-grain rice. The different varieties of rice samples were milled using a laboratory-scale milling machine with weight lever 1400 g at 5, 10, 20, 30 and 60 seconds. Arsenic contents in rice grain before and after milling were determined using Inductively Coupled Plasma-Mass Spectrometry. The results showed that arsenic contents in five varieties of long-grain rice decreased with increasing the degree of milling. Arsenic contents in rice samples were reduced about 20-30% after removing 6% of bran from brown rice and about 30-40% after removing 9-10% of bran from brown rice. The finding were in agreement with arsenic contents found in rice bran samples. The rice bran samples contained higher arsenic levels than those of polished rice and brown rice. Thus, rice bran should not be used for production of everyday consumed foods since it may pose risk of high exposure to arsenic in consumers who eat the products heavily and regularly.

KEYWORDS: ARSENIC, BROWN RICE, POLISHED RICE, MILLING

1 INTRODUCTION

Arsenic (As) is a metalloid found in the environment from natural processes and human activities. The long time arsenic exposure increased risk of cancer and chronic disease (EFSA, 2009; Fowler *et al.,* 2015; IARC, 2012). Rice *(Oryza sativa L.)* can absorb arsenic much more effectively than wheat and barley (Awasthi *et al.,* 2017). The wetland plants with submerged soil of rice roots are anaerobic conditions are referring to as redial oxygen loss of the rhizosphere, leading to arsenic contents absorb by root cell to rice grain. Arsenic accumulation in rice depends on arsenic contents in soil and water used for cultivation, agricultural input (pesticides and fertilizer contained arsenic compounds) and rice varieties (Awasthi *et al.,* 2017; Carey *et al.,* 2010; Islam *et al.,* 2016; Zhao, 2010). The natural variations of arsenic in soil related to arsenic distribution in rice grain. Arsenic contents in rice grain are problematic even where soil arsenic is at background levels (Kabir *et al*., 2016). Arsenic in rice grain may be presented at high concentrations in some contaminated crops.

Some previous studies found that rice husk had arsenic content higher than those of brown rice and polished rice, and the brown rice had higher arsenic levels than polished rice (Meharg *et al*., 2008; Ruangwises *et al.,* 2012; Seyfferth *et al*., 2011; Sun *et al*., 2008). Arsenic contents are much higher in bran layers than endosperm (Lombi *et al.,* 2009). The milling process is highly desirable to reduce arsenic contents in rice grain (Naito *et al*., 2015). However, effect of milling on the reduction of arsenic content in rice grain was depended on the varieties of rice (Naito *et al.,* 2015; Narukawa *et al.,* 2014; Sun *et al.,* 2008). The purpose of this study is to investigate effect of polishing on the removal of arsenic contents in five varieties of Thai long-grain rice.

2 MATERIALS AND METHODS

2.1 Sample Collection

Five varieties of paddy rice (two varieties of non-pigmented rice and three varieties of pigmented rice) collected from two contaminated crops that had found high arsenic contents in rice grain (total arsenic contents in brown rice > 0.35 mg/kg).

2.2 Sample Preparation

Five varieties of paddy rice (8 samples) which their moisture contents less than 14% were removed of husk using small rubber roll machine and separated the husk from the brown rice samples before milling (NgekSengHuat Co.,Ltd.). The impurity was separated from brown rice, and non-pigmented rice were separated from pigmented rice.

Milling: Eight brown rice samples (180 g) were milled to remove all or part of the bran layers using a laboratory-scale milling machine (friction action) with weight lever 1400 g (T.S.M. Science Co.,Ltd.). Each rice sample was milled at five different durations of time including 5, 10, 20, 30 and 60 seconds. Six-replicate experiments were conducted for each milling time. Furthermore, the rice bran was removed from brown rice at 30 seconds of milling, and collected for total arsenic contents determination.

2.3 Measurement of Degree of Milling for Rice

All polished rice samples were brushed to separated small impurity and sifted out the debris through hand sifter. Then, rice grain before milling or brown rice and polished rice were weighed for measure of the amount of bran removed from the brown rice calculated as the degree of milling (DOM). The broken rice was separated from the whole grain and head rice by grain separator (Thai Hua Chieng (1696) Co., Ltd.) and collected 150g of whole grain and head rice for determining total arsenic contents.

2.4 Total Arsenic Contents Determination

Rice powder and bran sample 1 g was transferred to a 50 mL polytetrafluoroethylene (PTFE) vessels, added 7 ml of concentrated nitric acid (HNO_3), placed in the fume hood at 25℃ and added 1 ml of 30% Hydrogen peroxide (H_2O_2) into the vessel and mixed. The vessels put in the water bath at 95 ℃ for 2 hours. Then, the vessels were cooled down to the room temperature, filter the digested solution into volumetric flask and added DI water to reach calibration mark of 25 ml. Finally, transfer the filtrate into a screw vial, as a test portion for determination of total arsenic by the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) according to AOAC 2013.06. (Julshamn *et al.*, 2013)

2.5 Statistical Analysis

Statistical analysis was conducted by SPSS Statistics Version 19.0. The significant differences of arsenic contents between brown rice and polished rice resulting from the process of polishing were tested. One-way analysis of variance (ANOVA) with LSD post-hoc test used to determine the effects of polishing on arsenic contents.

3 RESULTS

Total arsenic contents in brown rice samples were higher than those of polished rice. The highest total arsenic level was found in brown rice of pigmented rice namely PA 01 rice at the level of 0.74 mg/kg. The contents of total arsenic in polished rice samples with different time of milling indicated that total arsenic contents decreased, depending on degree of milling or loss of bran and germ.

Table 1: Degree of milling, total arsenic contents (expressed arsenic mg/kg wet weight) and reduction of arsenic contents in non-pigmented rice and bran using laboratory-scale milling machine (friction action) with weight lever 1400 g.

Sample	Time of milling (s)	Degree of milling (%)	Total Arsenic (mg/kg)	Reduction of Total Arsenic (%)
NPA 01 rice	0	-	0.47 ± 0.03^{a}	0.00^{a}
	5	3.34 ± 0.16	0.42 ± 0.03^{b}	10.71 ± 7.26^{b}
	10	6.14 ± 0.11	0.34 ± 0.01^{c}	28.21 ± 2.95^{c}
	20	8.08 ± 0.01	0.31 ± 0.01^{d}	34.29 ± 2.21^{d}
	30	9.21 ± 0.07	0.29 ± 0.01^{de}	37.50 ± 1.61^{de}
	60	11.79 ± 0.05	0.28 ± 0.01^{e}	40.00 ± 1.92^{e}
NPA 02 rice	0	-	0.43 ± 0.01^{a}	0.00^{a}
	5	3.22 ± 0.13	0.39 ± 0.01^{b}	10.00 ± 1.46^{b}
	10	5.84 ± 0.08	0.34 ± 0.01^{c}	21.54 ± 1.46^{c}
	20	8.23 ± 0.19	0.32 ± 0.01^{d}	25.77 ± 2.70^{d}
	30	9.57 ± 0.04	0.30 ± 0.01^{e}	30.00 ± 2.79^{e}
	60	12.32 ± 0.24	0.28 ± 0.01^{f}	36.54 ± 1.93^{f}
NPB 01 rice	0	-	0.38 ± 0.01^{a}	0.00^{a}
	5	3.48 ± 0.12	0.35 ± 0.02^{b}	8.77 ± 4.90^{b}
	10	6.19 ± 0.09	0.31 ± 0.00^{c}	18.86 ± 1.07^{c}
	20	7.98 ± 0.03	0.31 ± 0.02^{c}	19.74 ± 3.99^{c}
	30	9.06 ± 0.05	0.29 ± 0.01^{d}	24.56 ± 2.72^{d}
	60	12.71 ± 0.14	0.28 ± 0.02^{d}	26.32 ± 4.40^{d}
NPB 02 rice	0	-	0.55 ± 0.05^{a}	0.00^{a}
	5	3.55 ± 0.06	0.50 ± 0.03^{b}	10.54 ± 5.57^{b}
	10	6.43 ± 0.04	0.41 ± 0.04^{c}	25.60 ± 6.71^{c}
	20	8.71 ± 0.05	0.37 ± 0.01^{d}	33.73 ± 2.19^{d}
	30	9.83 ± 0.08	0.33 ± 0.01^{e}	40.36 ± 1.62^{e}
	60	13.38 ± 0.07	0.32 ± 0.01^{e}	43.07 ± 0.99^{e}
Bran NPA 01	30	9.21 ± 0.07	1.69	-
Bran NPA 02	30	9.57 ± 0.04	1.45	-
Bran NPB 01	30	9.06 ± 0.05	1.44	-
Bran NPB 02	30	9.83 ± 0.08	1.26	-

* NPA 1 rice and NPA 2 rice is the same variety of rice but different collected crops, including NPB 1 rice and NPB 2 rice.

**For degree of milling, total arsenic content and reduction of total arsenic content values represent arsenic mean ± SD, n=6

^{a}, ^{b}, ^{c}, ^{d}, ^{e}, and ^{f} in the same column with the same letter shows non-significant difference among time of milling for each sample code by One-way ANOVA *(P-value <0.05)*.

After milling non-pigmented rice with different time of milling, it was found that the total arsenic contents in the polished rice at the setting time of milling were reduced significantly from the brown rice. On the other hand, there was no significant difference of total arsenic contents between the polished rice at 30 and 60 seconds except NPA 02 rice sample (Table 1). The NPA 01 and NPA 02 rice samples were the same variety of non-pigmented rice but different collected crops. However, the total arsenic contents of NPA 01 and NPA 02 rice samples were nearly in brown rice and polished rice at the same time of milling (Figure 1a). The total arsenic contents in polished rice after removing 3%, 6%, 8%, 9–10% and 12% of bran were reduced approximately 10–11%, 22–28%, 26–34%, 30–38% and 37–40% of those found in brown rice, respectively (Table 1).

The NPB 01 and NPB 02 rice samples were the same variety of non-pigmented rice with different collected crops. The total arsenic contents of NPB 02 rice were higher than those of the NPB 01 rice in brown rice and polished rice at the same time of milling (Figure 1b). In addition, the percentages reduction of total arsenic contents of NPB 02 rice were higher than those of the polished rice of NPB

01 rice. However, the percentages reduction of arsenic contents in the polished rice of NPB 01 and NPB 02 at 30 and 60 seconds milling of time were not significantly different (Table 1).

Table 2: Degree of milling, total arsenic contents (expressed arsenic mg/kg wet weight) and reduction of arsenic contents in pigmented rice and bran using laboratory-scale milling machine (friction action) with weight lever 1400 g.

Sample	Time of milling (s)	Degree of milling (%)	Total Arsenic (mg/kg)	Reduction of Total Arsenic (%)
PA 01 rice	0	-	0.74 ± 0.04^{a}	0.00^{a}
	5	1.92 ± 0.03	0.72 ± 0.03^{a}	3.38 ± 3.60^{a}
	10	5.20 ± 0.13	0.61 ± 0.01^{b}	18.02 ± 1.85^{b}
	20	8.41 ± 0.03	0.49 ± 0.04^{c}	34.23 ± 5.90^{c}
	30	9.21 ± 0.02	0.46 ± 0.02^{c}	37.39 ± 2.37^{d}
	60	10.80 ± 0.10	0.46 ± 0.01^{c}	38.51 ± 1.42^{d}
PA 02 rice	0	-	0.36 ± 0.02^{a}	0.00^{a}
	5	2.44 ± 0.14	0.35 ± 0.01^{a}	2.78 ± 3.93^{a}
	10	6.18 ± 0.13	0.27 ± 0.01^{b}	25.46 ± 3.69^{b}
	20	8.92 ± 0.03	0.22 ± 0.01^{c}	38.43 ± 3.25^{c}
	30	9.68 ± 0.06	0.21 ± 0.01^{c}	41.20 ± 2.09^{c}
	60	12.16 ± 0.06	0.19 ± 0.01^{d}	46.30 ± 2.27^{d}
PB 01 rice	0	-	0.54 ± 0.02^{a}	0.00^{a}
	5	2.63 ± 0.06	0.48 ± 0.02^{b}	10.84 ± 4.24^{b}
	10	6.62 ± 0.12	0.41 ± 0.03^{c}	24.15 ± 5.31^{c}
	20	9.98 ± 0.02	0.36 ± 0.02^{d}	32.51 ± 3.03^{d}
	30	11.20 ± 0.07	0.35 ± 0.02^{de}	35.29 ± 3.41^{de}
	60	14.16 ± 0.04	0.34 ± 0.01^{e}	37.15 ± 2.73^{e}
PC 01 rice	0	-	0.38 ± 0.02^{a}	0.00^{a}
	5	4.69 ± 0.16	0.33 ± 0.01^{b}	14.10 ± 2.21^{b}
	10	8.40 ± 0.12	0.28 ± 0.01^{c}	26.43 ± 3.09^{c}
	20	12.21 ± 0.03	0.24 ± 0.01^{d}	37.89 ± 3.64^{d}
	30	14.50 ± 0.10	0.23 ± 0.01^{d}	39.21 ± 1.67^{de}
	60	20.09 ± 0.12	0.22 ± 0.01^{d}	41.85 ± 2.90^{e}
Bran PA 01	30	9.21 ± 0.02	2.51	-
Bran PA 02	30	9.68 ± 0.06	1.18	-
Bran PB 01	30	11.20 ± 0.07	1.39	-
Bran PC 01	30	14.50 ± 0.10	0.76	-

* PA 1 rice and PA 2 rice is the same variety of rice but different collected crops.

**For degree of milling, total arsenic content and reduction of total arsenic content values represent arsenic mean ± SD, n=6

[a], [b], [c], [d], [e], and [f] in the same column with the same letter shows non-significant difference among time of milling for each sample code by One-way ANOVA *(P-value <0.05)*.

The pigmented rice samples arsenic shown in the Table 2, after milling at different time of milling resulted that the total arsenic contents in the polished rice at the setting time of milling were reduced significantly from the brown rice. While the polished rice at 5 seconds time of milling of the PA 01 and PA 02 rice, it was found that total arsenic contents were no significantly different compared with those of the brown rice. In addition, there was no significant difference of total arsenic contents between the polished rice at 20 and 30 seconds (Table 2). The PA 01 rice and PA 02 rice samples were the same rice variety but different collected crops. The total arsenic contents of PA 01 rice were higher than those of the PA 02 rice in brown rice and polished rice at the same time of milling (Figure 1c). While in polished rice, the percentages reduction of total arsenic contents of PA 02 rice were higher than those of the PA 01 rice. The results showed that percentages reduction of total arsenic contents of the same rice variety namely PB 01 rice and PB 02 rice were different. However, the degree of milling of PA 02 rice was higher than the PA 01 rice at same time of milling. The total arsenic contents in polished rice

of PA 01 rice and PA 02 rice after removing about 2%, 5–6%, 8–9%, 9–10% and 11–12% of bran were reduced about 3%, 18–25%, 34–38%, 37–41% and 39–46% of those in brown rice, respectively (Table 2).

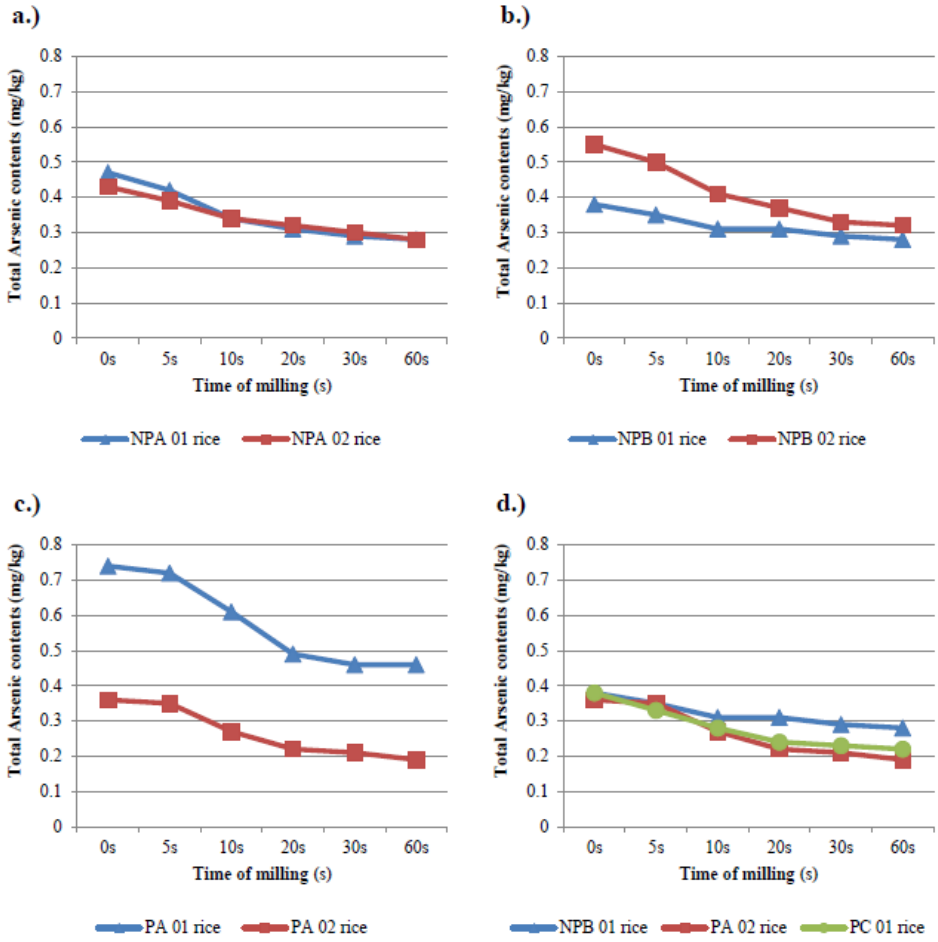


Figure 1: a.) The total As contents in the same rice variety and similar total As contents in brown rice but different collected crops with different time of milling using laboratory-scale milling machine (friction action) with weight lever 1400 g.

b.) The total As contents in the same variety of non-pigmented rice but different total As contents in brown rice and different collected crops with different time of milling using laboratory-scale milling machine (friction action) with weight lever 1400 g.

c.) The total As contents in the same pigmented rice variety but different total As contents in brown rice and different collected crops with different time of milling using laboratory-scale milling machine (friction action) with weight lever 1400 g.

d.) The total As contents in different varieties of rice but similar total As contents in brown rice with different time of milling using laboratory-scale milling machine (friction action) with weight lever 1400 g.

In the PB 01 and PC 01 rice samples, after milling with different time of milling resulted that the total arsenic contents in the polished rice at the setting time of milling was reduced significantly from those of the brown rice. On the other hand, there was no significant difference of total arsenic contents between the polished rice at 30 and 60 seconds (Table 2). The total arsenic contents in polished rice of PB 01 rice and PC 01 when increased removing of bran resulting in the increased reduction of total arsenic contents in brown rice.

From the result, three rice samples with different varieties namely NPB 01, PA 02 and PC 01 had similar total arsenic contents (Table 1 and 2). The reduction of total arsenic contents of polished rice of pigmented rice namely PA 02 and PC 01 were higher than those of polished rice of non-pigmented rice namely NPB 01 (Figure 1d).

Furthermore, the finding was in agreement with total arsenic contents found in rice bran samples after removing at 30 seconds time of milling or 9–14% removing bran of brown rice. The rice bran of all samples contained higher total arsenic contents than those of polished rice and brown rice. The total arsenic contents in rice bran of non-pigmented rice and pigmented rice were 1.26–1.69 mg/kg and 0.76–2.52 mg/kg, respectively (Table 1 and 2).

4 DISCUSSION

The results in this study were similar to some previous studies reported that the contents of arsenic in brown rice were higher than those of polished rice. The arsenic contents identified by X-ray absorption near-edge spectroscopy (XANES) and HPLC-ICP-MS showed that higher levels of arsenic were found in rice bran than that of endosperm. Consequently, when removing bran of brown rice were increased, the percentages reductions of arsenic contents were also increased (Jackson & Punshon, 2015; Lombi *et al*., 2009; Seyfferth *et al*., 2011).

The reductions of arsenic contents in rice samples were different, which possibly because of the difference of thickness of bran layer in rice samples. Juliano (2016) reported the thicker of aleurone layers of short grains than that of long grains (Juliano, 2016). In addition, the arsenic contents accumulated in rice were related to arsenic contents in soil and water of growing region and agricultural conditions (Ahmed *et al*., 2011; Islam *et al*., 2016). The arsenic accumulation of different rice varieties showed that arsenic contents in hybrid varieties were higher than those of the non- hybrid varieties (Rahman *et al*., 2007).

The percentages reductions of arsenic contents after milling in this study were similar to the Chinese and Bangladeshi rice. After 7% removing bran of brown rice of Chinese and Bangladeshi rice, reduction of arsenic contents were 33–40% and 18–23%, respectively. In Chinese rice, after milling, it was found that brown rice with high arsenic content had higher percentages reductions than those of rice samples with low arsenic contents *(Sun et al.*, 2008). The results was in agreement with the NPB 01 and NPB 02 rice in the present study.

While some Bangladeshi rice samples were similar to the PA 01 rice, the low content of arsenic in brown rice showed higher reduction of arsenic content than rice with high arsenic contents after milling of brown rice (Sun *et al*., 2008). In addition, the percentages of reductions of total arsenic contents of Japanese rice were 16–24% and 34–39% after removing bran of brown rice at 5% and 10%, respectively (Naito *et al*., 2015).

These result suggests that total arsenic content is mostly located in the rice bran of brown rice, which supported the findings of previous studies that arsenic accumulated at the surface, in the pericarp and aleurone layer (Meharg *et al*., 2008). The previous studies found that the total arsenic contents of bran were 3-9 times higher than those of brown rice and polished rice in Thai, Japanese, Chinese and Bangladeshi rice (Naito *et al*., 2015; Ruangwises *et al*., 2012; Sun *et al*., 2008).

5 CONCLUSION

The milling process which remove rice bran could reduce total arsenic contents in five varieties of Thai long-grain rice. The decreases of arsenic contents in polished rice depended on the degree of milling because the higher of arsenic were found in rice bran than that of endosperm. Thus study found that inorganic arsenic contents in five varieties of Thai long-grain rice were complying with the Codex Standard for polished rice (0.20 mg/kg) (CODEX STAN 193-1995). When the removing bran of brown rice was increased, the reductions of arsenic contents also increased. The removal of approximately 10% of outer layers of the brown rice caused the highest reduction of As contents in polished rice afterwards the total arsenic contents was not or less reduced in polished rice. As contents in non-pigmented rice were reduced about 20-40% after removing 9-10% of bran from brown rice and about 32-38% after removing 9-12% of bran from brown rice in pigmented rice. However, the rice bran found higher arsenic contents than those of polished rice and brown rice. Thus, the rice bran should not be using for production of everyday consumed foods since it may pose risk of high exposure to arsenic in consumers who eat the products heavily and regularly.

6 ACKNOWLEDGEMENTS

This research was supported by a grant from National Research Council of Thailand (NRCT) and Agricultural Research Development Agency (Public Organization) (ARDA). We also thank Sakon Nakhon Rice Research Centre for facilitating of rice milling process.

REFERENCE

- Ahmed, Z., *et al*. (2011). Genotype and environment effects on rice (Oryza sativa L.) grain arsenic concentration in Bangladesh. *An International Journal on Plant-Soil Relationships, 338*, 367-382.
- Awasthi, S., Chauhan, R., Srivastava, S., & Tripathi, R. D. (2017). The Journey of Arsenic from Soil to Grain in Rice. *Frontiers in Plant Science, 8*, 1007.
- Carey, A.-M., Scheckel, K. G., Lombi, E., Newville, M., Choi, Y., Norton, G. J., Meharg, A. A. (2010). Grain Unloading of Arsenic Species in Rice. *Plant Physiology, 152*(1), 309.
- CODEX STAN 193-1995. General Standard for Contaminants and Toxins in Food and Feed. In: Committee on Contaminants in Foods (CCCF) (ed.), *Codex Alimentarius*(pp.45-46).Codex Alimentarius International Food Standard
- European Food Safety Authority (EFSA). (2009). Scientific Opinion on Arsenic in Food. *EFSA Journal, 7*(10), 1351.
- Fowler, B. A., Selene, C. H., Chou, R. J., Jones, D. L., Sullivan Jr, W., & Chen, C. J. (2015). Arsenic. In G.F. Nordberg, B.A. Fowler & M. Nordberg. *Handbook on the Toxicology of Metals* (4 ed., pp. 581-624). San Diego: Academic Press.
- Interantional Agency for Research on Cancer(IARC). *IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS*. (2012). http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C-6.pdf Accessed 24 July 2018
- Islam, S., Rahman, M. M., Islam, M. R., & Naidu, R. (2016). Arsenic accumulation in rice: Consequences of rice genotypes and management practices to reduce human health risk. *Environment International, 96*, 139-155.
- Jackson, B., & Punshon, T. (2015). Recent Advances in the Measurement of Arsenic, Cadmium, and Mercury in Rice and Other Foods. *Current environmental health reports, 2*(1), 15-24.
- Juliano B.O. (2016) Rice: Overview. In: Wrigley, C., Corke, H., and Seetharaman, K., Faubion, J., (eds.), *Encyclopedia of Food Grains vol. 1* (pp. 125-129). Oxford: Academic Press.
- Julshamn, K., Maage, A., Skaar Norli, H., H Grobecker, K., Jorhem, L., Fecher, P., & Dowell, D. (2013). Determination of Arsenic, Cadmium, Mercury, and Lead in Foods by Pressure Digestion and Inductively Coupled Plasma/Mass Spectrometry: First Action 2013.06. *Journal of AOAC International*, 96, 1101-1102.
- Lombi, E., Scheckel, K. G., Pallon, J., Carey, A. M., Zhu, Y. G., & Meharg, A. A. (2009). Speciation and distribution of arsenic and localization of nutrients in rice grains. *New Phytol, 184*(1), 193-201.
- Meharg, A. A., Lombi, E., Williams, P. N., Scheckel, K. G., Feldmann, J., Raab, A., . . . Islam, R. (2008). Speciation and Localization of Arsenic in White and Brown Rice Grains. *Environmental Science & Technology, 42*(4), 1051-1057.
- Naito, S., Matsumoto, E., Shindoh, K., & Nishimura, T. (2015). Effects of polishing, cooking, and storing on total arsenic and arsenic species concentrations in rice cultivated in Japan. *Food Chemistry, 168*, 294-301.
- Narukawa, T., Matsumoto, E., Nishimura, T., & Hioki, A. (2014). Determination of Sixteen Elements and Arsenic Species in Brown, Polished and Milled Rice. *Analytical Sciences, 30*(2), 245-250.
- Nookabkaew, S., Rangkadilok, N., Mahidol, C., Promsuk, G., & Satayavivad, J. (2013). Determination of Arsenic Species in Rice from Thailand and Other Asian Countries Using Simple Extraction and HPLC-ICP-MS Analysis. *Journal of Agricultural and Food Chemistry, 61*(28), 6991-6998.
- Rahman, M., Hasegawa, H., Mahfuzur Rahman, M., Nazrul Islam, M., Miah, M. A. M., & Tasmin, A. (2007). Arsenic Accumulation in Rice (Oryza sativa L.) Varieties of Bangladesh: A Glass House Study. *Water, Air, and Soil Pollution*, 185.

- Ruangwises, S., Saipan, P., Tengjaroenkul, B., & Ruangwises, N. (2012). Total and inorganic arsenic in rice and rice bran purchased in Thailand. *J Food Prot, 75*(4), 771-774.
- Seyfferth, A. L., Webb, S. M., Andrews, J. C., & Fendorf, S. (2011). Defining the distribution of arsenic species and plant nutrients in rice (Oryza sativa L.) from the root to the grain. *Geochimica et Cosmochimica Acta, 75*(21), 6655-6671.
- Sun, G.-X., Williams, P. N., Carey, A.-M., Zhu, Y.-G., Deacon, C., Raab, A., Meharg, A. A. (2008). Inorganic Arsenic in Rice Bran and Its Products Are an Order of Magnitude Higher than in Bulk Grain. *Environmental Science & Technology, 42*(19), 7542-7546.
- Zhao, F.-J. (2010). Arsenic as a Food Chain Contaminant: Mechanisms of Plant Uptake and Metabolism and Mitigation Strategies. *Annual review of plant biology, v. 61*, pp. 535-559-2010 v.2061. http://doi.org/10.1146/annurev-arplant-042809-112152

Effect of Boiling on Arsenic Contents and Antioxidant Activity in Thai Rice Noodle Product Incorporating Pigmented Broken Rice and Rice Bran

Sirirat Khundam, Wenika Benjapong*, Pornrat Sinchaipanit, Ratchanee Kongkachuichai, Weeraya Karnpanit, Yaowalak Rooppat, Jakkit Sakolkittinapakul

Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand
*Corresponding Author (wenika.ben@mahidol.ac.th)

ABSTRACT

Rice bran of pigmented rice is used for enrichment of healthy foods, due to its high antioxidant compounds. Development of Thai rice noodle (Khanom-jeen) incorporating pigmented broken rice and rice bran is one of the effective ways to add value to rice by-products. However, rice bran also contains high levels of arsenic. This study aimed to investigate the effect of boiling on arsenic content and antioxidant activity of Thai rice noodle incorporating pigmented broken rice and rice bran. Three formulations of Thai rice noodle were prepared from pigmented rice flour mixed with 0, 5 and 20% of pigmented rice bran. Arsenic contents in rice bran, mixed rice flour and cooked Thai rice noodle samples were determined by Inductively Coupled Plasma-Mass Spectrometry. Antioxidant activity of the samples were measured by 2,2-Diphenyl-1-picrylhydrazylradical scavenging capacity assay. The results indicated that arsenic contents and antioxidant activity of the rice flour samples increased with increasing amount of rice bran. After boiling, arsenic contents in Thai rice noodle samples with 0, 5 and 20% of pigmented rice bran were reduced by 34, 39 and 40%, respectively, while antioxidant activity decreased by 63, 59 and 93%. Boiling method could reduce arsenic contents in the finished rice noodle products. Nevertheless, it also decreased antioxidant activity in the products.

KEYWORD: Arsenic, Rice Bran, Thai Rice Noodle, Antioxidant Activity

1 INTRODUCTION

Pigmented rice is distinguished by the rice grain having red, black, brown, or purple in its pericarp, testa, and nucellus layer (Finocchiaro *et al.*, 2007; Yodmanee *et al*., 2011). Rice bran of pigmented rice naturally contained various bioactive compounds including phenolic acids and anthocyanins (Iqbal *et al*., 2005; Yawadio *et al.*, 2007; Zhang *et al*., 2006). Sompong *et al*., (2011) revealed that anthocyanins were the dominant compound in black and purple Thai rice, while phenolic acids were the major compounds in red Thai rice. Many of these bioactive compounds provided potential health-beneficial effects such as lowering total plasma cholesterol (Gerhardt and Gallo, 1998), reducing LDL cholesterol level (Hongu *et al.*, 2014), enhancement of immune system (Choi et al., 2007) and preventing cancer-cell invasion (Chen *et al.*, 2006). Goffman *et al.*, (2004) reported that the total phenolic contents in purple and red rice bran were approximately 10 times higher than that of light brown rice bran, indicating that antioxidant activity of rice bran closely related to rice bran color. Nowadays, rice bran has been broadly applied in food industry due to high nutritional values and rich of bioactive compounds. However, rice can accumulate higher amount of arsenic (As) than other crops, and arsenic is mainly accumulated in the husk and bran of rice (Meharg *et al.*, 2008; Williams *et al.*, 2005). A previous study reported that rice bran contained total arsenic (total As) concentrations approximately seven times higher than those found in the corresponding polished rice (Ruangwises *et al.*, 2012).

Arsenic, a toxic element and naturally occurred in water, air and soil, and is absorbed by some food crops. Long-term exposure to high level of arsenic is associated with higher rates of skin, bladder and lung cancers, as well as heart disease and diabetes (IARC, 2012). Referring to previous argument, fortification with rice bran has become popular in health-promoting value-added products, such rice bran fortified food tends to be elevated in arsenic. Therefore, arsenic contents in rice and rice bran-incorporated products should be reduced for safe consumption.

Several studies had suggested that cooking is one of the effective process to reduce arsenic contents in rice and rice bran. The effects of cooking on arsenic contents in rice depend on various factors such as (a) type of rice (brown or polished rice) (b) cooking method (steaming or parboiling) and (c) ratio of rice to water (Mihucz *et al.,* 2007; Sengupa *et al.,* 2006; Torres-Escribano *et al.,* 2008). Similarly to arsenic contents, these factors might affect the bioactive compounds contents in rice and rice bran. Traditional Thai rice noodle also called "Khanom-jeen" widely consumed in Thailand, is made by broken rice. The present study used pigmented rice bran as a functional ingredient to enhance the nutritive values of Thai rice noodle; however, the amount of arsenic contents in the product should be of concern. Therefore, this study aimed to investigate the effect of boiling on arsenic contents and antioxidant activity of Thai rice noodle incorporating pigmented broken rice and rice bran.

2 MATERIALS AND METHODS

2.1 Selection of Pigmented Rice Variety

Generally, pigmented rice varieties are polished with low milling degree to obtain under-milled rice with partial bran left. Therefore, rice variety with high total arsenic content and antioxidant activity in under-milled rice was selected to produce Thai pigmented rice noodle. Based on our preliminary study, three pigmented rice samples were collected from three arsenic contaminated crops in August 2016. Paddy rice samples were milled and removed husk by husk separator machine, brown rice samples were polished with low milling degree (under-milled rice) and high milling degree (well-milled rice) using laboratory-scale milling machine (friction action). Total arsenic contents and antioxidant activity of all samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and 1,1-Diphenyl-2-picrythydrazyl (DPPH) radical scavenging assay, respectively. Total arsenic contents and antioxidant activities of brown rice, under-milled rice and well-milled rice are shown in Table 1. The highest total arsenic content and antioxidant activity of under-milled rice were found in red pericarp variety. Consequently, this variety was selected to develop pigmented broken rice and rice bran incorporated Thai rice noodle.

Table 1: Total arsenic concentration and antioxidant activity in rice samples with different degree of millings (Harvested in August 2016)

Rice sample	Sample type	Degree of milling (%)	Total As (mg/kg)	Antioxidant activity DPPH (mg Trolox eqv./100g)
Red pericarp long grain rice	Brown rice		0.75 ± 0.05	327.25 ± 13.71
	Under-milled rice	5.25	0.60 ± 0.01	122.12 ± 6.71
	Well-milled rice	9.20	0.47 ± 0.01	42.21 ± 0.16
Dark red pericarp long grain rice	Brown rice		1.06 ± 0.03	403.61 ± 18.84
	Under-milled rice	6.60	0.42 ± 0.04	114.78 ± 1.88
	Well-milled rice	11.15	0.34 ± 0.03	33.08 ± 1.76
Black pericarp long grain rice	Brown rice		0.39 ± 0.02	85.23 ± 0.16
	Under-milled rice	8.40	0.47 ± 0.01	49.19 ± 0.40
	Well-milled rice	14.50	0.23 ± 0.01	33.34 ± 0.09

Data were expressed as mean ± standard deviation, based on wet weight basis.
* Degree of milling (DOM) = the percentage of outer layer removal from brown rice.

2.2 Sample Preparation

2.2.1 Preparation of Rice Flour

Red pericarp long-grain rice sample was collected from the same contaminated crops in August, 2017. Rice samples were milled and removed husk, brown rice samples were polished to under-milled rice and well-milled rice. The by-products of the rice milling included broken rice, under-milled rice bran and well-milled rice bran. The under-milled broken rice sample was ground by a grinder to a 100 micron particle size. The rice flour was sifted through ISS 40 metal sieve**.** The rice flour and its bran with high arsenic content and antioxidant activity were used for preparation of Khanom-jeen. Total arsenic contents and antioxidant activities of brown rice, broken rice and its bran are shown in Table 2.

Table 2: Arsenic content and antioxidant activity in brown rice, broken rice and rice bran of the red pericarp rice sample (Harvested in August 2017).

Rice type	Degree of milling (%)	Total As content (mg/kg)	Antioxidant activity DPPH (mg Trolox eqv. /100 g)
Brown rice		0.54 ± 0.02^{c}	297.90 ± 39.48^{c}
under-milled broken rice	4.87	0.51 ± 0.03^{c}	132.80 ± 3.63^{d}
Under-milled rice bran		2.84 ± 0.11^{a}	3658.28 ± 62.93^{a}
Well-milled rice bran		2.54 ± 0.067^{b}	2197.07 ± 145.58^{b}

Data were presented as means ± standard deviation, based on wet weight basis.
^{a,b,c} different letters within the same column indicates significantly different ($p < 0.05$) according to one way ANOVA followed by Least Significant Difference (LSD) test.

2.2.2 Preparation of Roasted Rice Bran

Rice bran of under milled red pericarp rice was cook using a common method used by rice bran producers. The bran was roasted on low heat for 5 minutes, until dark brown color and fragrant. Continuous stirring was performed to avoid burning.

Three rice flour formulas prepared from pigmented broken rice and bran (0, 5 and 20%) were used to develop the Thai rice noodle samples (Table 3). The maximum percentage amount of rice bran (20%) being mixed in the flour based on ability to hold shape of noodle after passing the dough through an extruder.

Table 3: Amounts of broken rice flour and bran used for preparing three formulations of Thai rice noodle

Formulations	Broken rice flour (g)	Rice bran (g)
A (0% rice bran)	300	0
B (5% rice bran)	285	15
C (20% rice bran)	260	40

2.2.3 Preparation of Thai Rice Noodle

Added deionized water (240 ml) into raw rice flour (300 g) and kneaded for 25 minutes to form a soft dough and molded into balls. The dough was boiled in water at 100 °C for 1 minute. The pre-gelatinized dough was re-kneaded to paste-like consistency with deionized water (150 ml). The dough was passed through noodle extruder into hot water (80-90 °C), then immediately cooled with water, drained, and desiccated for 30 minutes. Thai rice noodle sample was stored in refrigerator at -22 °C. Six-replicate experiments were performed.

2.3 Determination of Total Arsenic Content

Total arsenic contents of rice flour and Thai rice noodle were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) according to AOAC 2013.06. Rice powder 1 g was transferred to a 50 ml polytetrafluoroethylene (PTFE) vessels, 7 ml of concentrated nitric acid (HNO_3) was subsequently added to the vessel and placed in the fume hood at 25 °C. Added 1 ml of 30% hydrogen peroxide (H_2O_2) into the vessel, mixed, put it in the water bath at 95 °C for 2 hours and cooled down at room temperature. Digested solution was transferred and collected into 25 ml with deionized water. Finally, it was filtered and used for determination of total arsenic concentration.

2.4 Determination of antioxidant Activity

Antioxidant activity in rice sample with different degree of milling were evaluated through DPPH radical scavenging activity assay according to the method reported by Molyneux (2004). Briefly, dried grounded sample (1 g) was mixed with 9 ml of 80% methanol. Extraction was centrifuged for 5 min. DPPH reaction mixture was prepared by mixing 0.3 ml of DPPH radical solution (2 mM) with 3.7 ml of 80% methanol. To this 100 µl of extract was added at various dilutions. The absorbance was measured at 515 nm after 20 min standing at room temperature. The measurement of radical scavenging activity was done using Trolox as standards and the values are expressed as mg of Trolox equivalents/100 g, the concentration of the samples that causes 50% scavenging of DPPH radical.

Antioxidant activity in rice flour and Thai rice noodle were carried out as described by Katsube *et al*., (2004). After the extracts had been diluted with water stepwise, 10 μL of dilution was pipetted into a 96-well plate. 185 μL of DPPH solution dissolved in a 50% ethanol solution was added to each well, and the plate was shaken for 5 min at room temperature. Radical scavenging activity is shown as EGCG equivalent per 100 gram of sample (mg of EGCG equivalents /100 g) on the basis of the concentration for 50% inhibition of red coloration at 550 nm.

2.5 Statistical Analysis

The significant difference of total arsenic content and antioxidant activity among rice flour and Thai rice noodle were analyzed using one way analysis of variance (one- way ANOVA) with LSD post-hoc test. Independent-sample t-test was used to compare the arsenic contents and antioxidant activity between rice flour and Thai rice noodle. *P*-values of less than 0.05 were considered as statistically different using SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA).

3 RESULTS

3.1 Changes of Total Arsenic Contents in Rice Flour and Thai Rice Noodle after Boiling Conditions.

Total arsenic contents in rice flour and Thai rice noodle are shown in Table 4. Total arsenic contents in rice flour significantly increased with increasing amount of rice bran ($P \leq 0.05$). Rice flour formulation C contained 1.3 times of total arsenic content compared to rice flour without rice bran (formulation A). The mean values of total arsenic content were 0.63, 0.90, and 1.42 mg/kg (wet weight basis) for rice flour A, B, and C, respectively. Significant reductions in total arsenic concentration (dry weight basis) by 39, 40 and 34% of initial values of rice flour formulation A, B, and C, respectively were found after boiling Thai rice noodle.

Table 4: Total arsenic contents in rice flour and Thai rice noodle.

Sample	Wet weight basis		Dry weight basis	
	Total As (mg/kg)	Reduction of total As (%)	Total As (mg/kg)	Reduction of total As (%)
Roasted rice bran	3.60		3.79	
A (0% rice bran)				
Rice flour	$0.63 \pm 0.04^{c,A}$	81.15 ± 1.54^{x}	$0.71 \pm 0.04^{c,A}$	39.11 ± 4.12^{xy}
Thai rice noodle	$0.12 \pm 0.01^{c,B}$		$0.43 \pm 0.03^{c,B}$	
B (5% rice bran)				
Rice flour	$0.90 \pm 0.04^{b,A}$	80.45 ± 0.42^{x}	$1.02 \pm 0.05^{b,A}$	40.37 ± 3.20^{x}
Thai rice noodle	$0.18 \pm 0.01^{b,B}$		$0.62 \pm 0.02^{b,B}$	
C (20% rice bran)				
Rice flour	$1.42 \pm 0.06^{a,A}$	77.92 ± 1.73^{y}	$1.59 \pm 0.06^{a,A}$	34.56 ± 4.27^{y}
Thai rice noodle	$0.31 \pm 0.02^{a,B}$		$1.07 \pm 0.06^{a,B}$	

Data were expressed as mean ± standard deviation

^{a,b,c} different letters within the same column was significantly different ($p < 0.05$) in total arsenic content in each formula according to one way ANOVA followed by LSD test (Comparison within the group of flour or noodles).

^{A,B} different letters within the same column was significantly different ($p < 0.05$) in total arsenic content between rice flour and Thai rice noodle according to paired sample t-test.

^{x,y} different letters within the same column was significantly different ($p < 0.05$) in percentage reduction of total arsenic content in each formula according to one way ANOVA followed by LSD test.

3.2 Changes in Antioxidant Activity of Rice Flour and Thai Rice Noodle.

Rice Flour C showed the highest DPPH Radical Scavenging Activity (1569.96 ± 88.41 mg Trolox equivalent /100 g wet weight of sample). It appeared that the DPPH radical scavenging activity significantly increased with an increase in the proportion of pigmented rice bran. Changes in antioxidant activity in Thai rice noodle after boiling was examined (Table 5). The DPPH radical scavenging activity of Thai rice noodle A, B, and C were 58.52 ± 3.79, 113.90 ± 4.35, and 1569.96 ± 88.41, respectively. Compared with rice flour, the DPPH radical scavenging activity in Thai rice noodle after boiling was significantly decreased by 88, 86, and 98%, respectively ($P \leq 0.05$). Therefore, boiling had a dramatic effect on the antioxidant activity of Thai rice noodle incorporating rice bran.

Table 5: Antioxidant activities in rice flour and Thai rice noodle.

Sample	Wet weight basis		Dry weight basis	
	Antioxidant activity DPPH (mg ECGC eqv/100 g)	Reduction of antioxidant activity (%)	Antioxidant activity DPPH (mg ECGC eqv/ 100g)	Reduction of antioxidant activity (%)
Roasted rice bran	17164.21		18,041.04	
A (0% rice bran)				
Rice flour	$58.52 \pm 3.79^{c,A}$	88.81 ± 0.09^{y}	$66.16 \pm 4.30^{c,A}$	63.775 ± 3.281^{y}
Thai rice noodle	$6.54 \pm 0.53^{c,B}$		$23.89 \pm 1.67^{c,B}$	
B (5% rice bran)				
Rice flour	$113.90 \pm 4.35^{b,A}$	86.77 ± 1.12^{z}	$128.76 \pm 5.06^{b,A}$	52.623 ± 1.93^{z}
Thai rice noodle	$15.03 \pm 0.89^{b,B}$		$52.623 \pm 1.93^{b,B}$	
C (20% rice bran)				
Rice flour	$1569.96 \pm 88.41^{a,A}$	97.905 ± 0.18^{x}	$1759.15 \pm 99.47^{a,A}$	93.57 ± 0.58^{x}
Thai rice noodle	$32.810 \pm 2.20^{a,B}$		$112.797 \pm 6.20^{a,B}$	

Data were expressed as mean ± standard deviation

^{a,b,c} different letters within the same column was significantly different ($p < 0.05$) in antioxidant activity in each formula according to one way ANOVA followed by LSD test (Comparison within the group of flour or noodle).

^{A,B} different letters within the same column was significantly different ($p < 0.05$) in antioxidant activity between rice flour and Thai rice noodle according to paired sample t-test.

^{x,y,z} different letters within the same column was significantly different ($p < 0.05$) in percentage reduction of antioxidant activity in each formula according to one way ANOVA followed by LSD test.

4 DISCUSSION

4.1 Changes in Total Arsenic Content in Rice Flour and Thai Rice Noodle after Boiling Conditions.

Boiling with deionized water significantly reduced by 34-40% of total As content in Thai rice noodle. The results was consistent with the previous study reported that boiling rice with deionized water reduced the total As content of whole grain rice (Laparra *et al*., 2005). According to the previous study and present study, rice or rice product samples were boiled in excess water and the cooking water was discarded. Therefore, the boiling method can partially reduce As concentration in cooked rice or rice products. This is primarily caused by heat treatment accelerates the breaking of bounds between arsenic and the food matrix led to water-soluble arsenic compound which was released from cooked rice into the water at high temperature (100 °C) (Cheyns *et al*., 2017; Hajeb *et al.*, 2014; Rahman *et al*., 2006; Sengupta *et al.*, 2006). Interestingly, Thai rice noodle with 20% rice bran had the lower percentage reduction than that of Thai rice noodle with 5% rice bran, probably related to arsenic species in pigmented rice bran. For instance, rice bran had greater proportion of arsenate (As(V)) to arsenite (As (III)) than that of the broken rice flour. Arsenate had lower solubility than arsenite resulted in slightly reduced of total As in Thai rice noodle incorporated with 20% of rice bran after boiling (Smedley *et al*., 2004). In addition, the composition of rice bran might affect the reduction of arsenic

content. Rice bran contains phytic acid which has a strong ability to bind with heavy metals resulting in hardly released of arsenic from rice bran into water (Friedman *et al*., 2013; Hajeb *et al.,* 2014; Tsao *et al.,* 1997). In addition, minerals and other compounds in rice bran may bind with arsenic and has effect on the solubilization of arsenic in the cooking water (Cheyns *et al*., 2017; Rahman *et al*., 2006). Further research is needed to investigate the effect of compounds in rice that could bind arsenic and the relationship between the amount of As release and the structure of the foodstuff. Moreover, inorganic arsenic (i-As) in Thai rice noodle samples were not exceed the Commission Regulation (EC) No 1881/2006 standard for i-As in rice products at 0.3 mg/kg (data not shown).

4.2 Change in Antioxidant Activity of Rice Flour and Thai Rice Noodle.

In this study, antioxidant activity in pigmented rice bran was extremely higher than those of broken rice flour. This result indicated that antioxidant compounds were located in rice bran, which supports the finding of Moongngarm *et al.,* (2012); Orthoefer *et al.,* (2004) that antioxidant compounds were localized at the surface, in the region corresponding to the pericarp, testa, nucellus, and aleurone layer. Previous data showed that the antioxidant activity of pigmented rice bran was highly correlated with phenolic acid and anthocyanin (subgroup of flavonoids) (Goffman *et al.,* 2004; Oki *et al.,* 2002). Thus, phenolic acid and anthocyanin might be the major functional compounds responsible for antioxidant activity in rice bran in this study.

It was observed that the antioxidant activity of Thai rice noodle decreased drastically up to 93% after boiling which was probably due to the decline of phenolic acid and anthocyanin levels in Thai rice noodle during boiling (Zhang *et al*., 2010a) as these compounds are the main components responsible for the antioxidant activity of pigmented rice (Kaneda *et al.,* 2007; Surh *et al.,* 2014; Walter *et al*., 2013; Yamuangmorn *et al*., 2018). Massaretto (2011) found that the soluble phenolic acids in pigmented rice dropped by 83% after cooking, indicating that the soluble phenolic fraction, mainly composed of anthocyanins, was the most affected by the thermal treatment. Finocchiaro *et al*., (2007) observed that 70% and approximately 96% of the anthocyanin in red rice were lost when grains were cooked in an exact amount of water (complete absorption) and in excess water, respectively. Therefore, we presumed that losses can be vastly variable, if cooking conditions by using an excess of water permit the leaching OF water soluble compounds. Some previous studies reported that cooking under temperature about 114 °C degraded phenolic compounds and anthocyanin as well as leaching of their soluble compounds into the cooking water (Melini *et al.,* 2017; Randhir *et al*., 2008; Zaupa *et al.,* 2015; Zhang *et al.,* 2010b). Moreover, other components of rice including amylose, fat, and protein could form inclusion complexes with antioxidant compounds, and also affect on water absorption and compound leaching in rice and rice bran.

5 CONCLUSION

This study showed that incorporating rice bran into flour enhanced antioxidant activity; however, arsenic content in the flour was also raised. Increasing amount of rice bran in the formulation resulting in higher arsenic contents and antioxidant activity. After boiling Thai rice noodles, arsenic contents were reduced by 34-40% depended on the formulations of rice bran incorporation. Significant reductions in antioxidant activity (63-93%) of the Thai rice noodles developed were found as a result of boiling. Boiling method was one of the effective ways to reduce arsenic contents in rice products; however, it also reduced antioxidant activity dramastically. Further study should vary cooking methods to investigate more effective way of reducing arsenic contents as well as preserving bioactive compounds in rice product. Consumer preference and sensory evaluation of rice bran incorporated rice noodled should be further studied.

REFERENCES

- Chen, P.-N., Kuo, W.-H., Chiang, C.-L., Chiou, H.-L., Hsieh, Y.-S., & Chu, S.-C. (2006). Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. *Chemico-Biological Interactions*, 163(3), 218–229.

- Cheyns, K., Waegeneers, N., Van de Wiele, T., & Ann Ruttens, A. (2017). Arsenic Release from Foodstuffs upon Food Preparation. Journal of Agricultural and Food Chemistry, 65(11), 2443-2453.
- Choi Y., Jeong H. S., & Lee, J. (2007). Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food Chemistry*, 102(1), 130-138.
- Finocchiaro, F., Ferrari, B., Gianinetti, A., Dall'Asta, C., Galaverna, G., Scazzina, F., & Pellegrini, N. (2007). Characterization of antioxidant compounds of red and white rice and changes in total antioxidant capacity during processing. *Molecular Nutrition & Food Research*, 51, 1006 – 1019
- Friedman, M. (2013). Rice brans, rice bran oils, and rice hulls: Composition, food and industrial uses, and bioactivities in humans, animals, and cells. *Journal of Agricultural and Food Chemistry*, **61**(45), 10626–10641.
- Gerhardt, A. L., & Gallo, N. B. (1998). Full-fat rice bran and oat bran similarly reduce hypercholesterolemia in humans. *Journal of Nutrition*, 128(5), 865-9.
- Goffman, F. D., & Bergman, C. J. (2004). Rice kernel phenolic content and its relationship with antiradical efficiency. *Journal of the Science of Food and Agriculture*, 84(110), 1235–1240.
- Hajeb, P., Sloth, J. J, Shakibazadeh, S., Mahyudin, N. A., & Afsah- Hejri, L. (2014). Toxic elements in food: occurrence, binding, and reduction approaches. *Comprehensive Reviews in Food Science and Food Safety*, 13(4), 457−472.
- Hongu, N., Kitts, D. D., Zawistowski, J., Dossett, C. M., Kopec, A., Pope, B. T., & Buchowski, M. S. (2014). Pigmented rice bran and plant sterol combination reduces serum lipids in overweight and obese adults. *Journal of the American College of Nutrition*, 33(3), 231-238.
- International Agency for Research on cancer (2012). Arsenic and arsenic compounds. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. *Arsenic, Metals, Fibres, and Dusts, Volumn 100c* (pp. 41-94).
- Iqbal, S., Bhanger, M. I., & Anwar, F. (2005). Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. *Food Chemistry*, 93(12) , 265–272.
- Julshamn, K. , Maage, A., Norli, H.S., Grobecker, K.H., Jorhem, L., Fecher, P., & Dowell, D. (2013). Determination of arsenic, cadmium, mercury, and lead in foods by pressure digestion and inductively coupled plasma/ mass spectrometry: first action 2013.06. Journal of AOAC INTERNATIONAL, 96(5), 1101-1102.
- Kaneda, I., Iwai, K., Kubo, H., & Sakurai, H. (2007). Relationship between trace metal concentration and antioxidative activity of ancient rice bran (red and black rice) and a present-day rice bran (Koshihikari). *Journal of Trace Elements in Medicine and Biology*, **21(1),** 43–51.
- Katsube, T., Tabata, H., Ohta, Y., Anuurad, E., Shiwaku, K. & Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *Journal of Agricultural and Food Chemistry*, 52(8), 2391-2396.
- Laparra, J. M., Vélez, D., Barberá, R., Farré, R., & Montoro, R. (2005). Bioavailability of inorganic arsenic in cooked rice: practical aspects for human health risk assessments. *Journal of Agricultural and Food Chemistry*, 53(22), 8829-8833.
- Massaretto, L. I., Madureira Alves, M. F., Mussi de Mira, N. V., Karaogla- novic Carmona, A., & Lanfer Marquez, U. M. (2011). Phenolic compounds in raw and cooked rice (*Oryza sativa* L.) and their inhibitory effect on the activity of angiotensin I-converting enzyme. *Journal of Cereal Science*, 54, 236–240.
- Meharg, A.A., Lombi, E., Williams, P.N., Scheckel, K.G., Feldmann, J., Raab, A., Zhu, Y., & Islam, R. (2008). Speciation and localization of arsenic in white and brown rice grains. *Environmental Science and Technology*. 42(4) , 1051–1057.
- Melini, V., & Acquistucci, R. (2017). Health-Promoting Compounds in Pigmented Thai and Wild Rice. *Foods*, 6(1), 2-13.
- Mihucz, V. G., Tatar, E., Virag, I., Zang, C., Jao, Y., & Zaray, G. (2007). Arsenic removal from rice by washing and cooking with water. *Food Chemistry*, 105(4), 1718–1725. https://doi.org/10.1016/j.foodchem.2007.04.057
- Molyneux, P. (2004) The Use of Stable Free Radical Diphenylpicrylhydrazyl (DPPH) for Estimating Antioxidant Activity. *Songklanakarin Journal of Science and Technology*, 26, 211-

219.https://www.researchgate.net/deref/http%3A%2F%2Fwww.sjst.psu.ac.th%2Fjournal%2F26-2.pdf%2F07-DPPH.pdf

- Moongngarm, A., Daomukda, N., & Khumpika, S. (2012). Chemical Compositions, Phytochemicals, and Antioxidant Capacity of Rice Bran, Rice Bran Layer, and Rice Germ. *APCBEE Procedia*, 3 – 79.
- Oki,T., Masuda, M., Kobayashi, M., Nishiba,Y., Furuta, S., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red- hulled rice, *Journal of Agricultural and Food Chemistry*, 50, 7524 – 7529.
- Orthoefer, F. T., & Eastman, J. (2004). Rice bran and oil. In E. T. Champagne (Ed.), Rice: *Chemistry and technology*, (3rd ed., pp. 569–593). Minnesota: American Association of Cereal Chemists Inc.
- Rahman, M.A., Hasegawa, H., Rahman, M.A., Rahman, M.M., & Miah, M. A. M. (2006). Influence of cooking method on arsenic retention in cooked rice related to dietary exposure. *Science of the Total Environment*, 370(1), 51–60.
- Randhir, R., Kwon, Y. & Shetty, K. (2008). Effect of thermal processing on phenolics antioxidant activity and health relevant functionality of select grain sprouts and seedlings. *Innovative Science & Emerging Technology*, 9(3), 355-364.
- Ruangwises, S., Saipan, P., Tengiaroenkul, B., & Ruangwises, N. (2012). Total and inorganic arsenic in rice and rice bran purchased in Thailand. *Journal of Food Protection*, 75(4), 771-774.
- Sengupta, M. K., Hossain, M. A., Mukherjee, A., Ahamed, S., Das, B. Nayak, B., Pal, A., & Chakraborti, D. (2006). Arsenic burden of cooked rice: traditional and modern methods. *Food and Chemical Toxicology*, 44(1), 823-1829.
- Smedley, P. L., & Kinniburgh, D. G. (2002). A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry*, 17(5): 517-568.
- Sompong, R., Siebenhandl-Ehn, S., Linsberger-Martin, G., & Berghofer, E. (2011). Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. *Food Chemistry*, 124(1), 132–140.
- Surh, J., & Koh, E. (2014). Effects of four different cooking methods on anthocyanins, total phenolics and antioxidant activity of black rice. *Journal of the Science of Food and Agriculture*, 94(15), 3296–3304.
- The commission's regulation (EU) 2015/1006 of 25 June 2015 on amending regulation (EC) 1881/2006 regarding maximum levels for inorganic arsenic in food stuffs. Official Journal of the European Union L 161, pp. 14-16. http://www.fao.org/faolex/results/details/en/c/LEX-FAOC146756/ Accessed 18 August 2018
- Torres-Escribano, S., Leal, M., Vélez, D. & Montoro, R. (2008). Total and inorganic arsenic concentrations in rice sold in Spain, effect of cooking, and risk assessments. *Environmental Science and Technology*, 42(10), 3867–3872.
- Tsao, G. T., Zheng, Y., Lu, J., & Gong, C. S. (1997). Adsorption of heavy metal ions by immobilized phytic acid. *Applied biochemistry and biotechnology*, 63-65, 731-41.
- Walter, M., Marchesan, E., Massoni, P. F. S., da Silva, L. P., Sartori, G. M. S., & Ferreira, R. B. (2013). Antioxidant properties of rice grains with light brown, red and black pericarp colors and the effect of processing. *Food Research International*, 50, 698–703.
- Williams, P. N., Price, A. H., Raab, A., Hossain, S. A., Feldmann, J., & Meharg, A. A. (2005). Variation in arsenic speciation and concentration in paddy rice related to dietary exposure. *Environmental Science and Technology*, 39(15), 5531–5540.
- Yamuangmorn, S., dELL, B., & Prom- U- Thai, C. (2018). Effects of Cooking on Anthocyanin Concentration and Bioactive Antioxidant Capacity in Glutinous and Non-Glutinous Purple Rice. *Rice Science*, 2018, 25(5), 270-278.
- Yawadio, R., Tanimori, S., & Morita, N. (2007). Identification of phenolic compounds isolated from pigmented rices and their aldose reductase inhibitory activities. *Food Chemistry*, 101 (4), 1616-1625.
- Yodmanee, S., Karrila, T.T. & Pakdeechanuan, P. (2011). Physical, chemical and antioxidant properties ofpigmented rice grown in Southern Thailand. *International Food Research Journal*, 18, 901– 906.

- Zaupa, M., Calani, L., Del Rio, D., Brighenti, F., & Pellegrini, N. (2015). Characterization of total antioxidant capacity and (poly) phenolic compounds of differently pigmented rice varieties and their changes during domestic cooking. *Food Chemistry*, 187, 338–347.
- Zhang, M. W., Guo, B. J., Zhang, R. F., Chi, J. W., Wei, Z. C., & Xu, Z. H. (2006). Separation, purification and identification of antioxidant compositions in black rice. A*gricultural Sciences in China*, 5(6), 431–440.
- Zhang, M. W., Zhang, R. F., Zhang, F. X., & Liu, R.H. (2010a). Phenolic profiles and antioxidant activity of black rice bran of different com- mercially available varieties. *Journal of Agricultural and Food Chemistry*, 58, 7580- 7587.
- Zhang, M., Chen, H., Li, J., Pei, Y., & Liang, Y. (2010b). Antioxidant properties of tartary buckwheat extracts as affected by different thermal processing methods. *LWT-Food Science and Technology*, 43(1), 181-185.

Inadequate intake of Dietary Fiber, Vitamin B_{12}, and Calcium in Thai Elders

Budtree Treesattayakul[1]*, Thunnalin Winuprasith[2], Budsakorn Teeranurak[3], Dunyaporn Trachootham[2]

[1] Master Program in Nutrition and Dietetics, Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand
[2] Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand
[3] Department of Dental Service, Phuttamonthon Hospital, Nakhon Pathom, Thailand
*Corresponding Author (budtree.tre@hotmail.com)

ABSTRACT

Objective: This study aimed to investigate adequacy of dietary fiber, vitamin B_{12}, and calcium intakes in Thai older adults. **Design:** This observational study used cross-sectional design. **Setting:** Data were collected at Institute of Nutrition, Mahidol University, Thailand. **Participants:** Ninety Thai healthy older adults aged 51-79 years old were recruited. **Measurements:** Dietary fiber, vitamin B_{12}, and calcium intakes were assessed by 4-day food record. Adequacy of nutrient intakes were analyzed and compared with the Thai Dietary Reference Intake (Thai DRI) by using INMUCAL-Nutrients. Consumption frequencies of green leaves, meat, and dairy product were measured by using food frequency questionnaire (FFQ). **Results:** 100% of participants had inadequate intake of dietary fibers. Consistently, up to 55% of participants consumed green leaf and yellow vegetables only once a week. While up to 20% of participants did not consume vegetables at all. Furthermore, inadequate calcium intake was found in 90% of participants. Likewise, there were over 70% of participants having inadequate intake of vitamin B_{12}. Consistently, up to 30% of participants consumed milk or meat only once a week. While up to 40% did not consume milk or meat at all. **Conclusion:** our findings suggested that most Thai older adults inadequately consumed dietary fiber, vitamin B_{12} and calcium. Development of food product for elderly should consider fortification of these nutrients.

KEYWORDS: Dietary Fiber, Vitamin B12, Calcium, Older Adults

1 INTRODUCTION

Currently, the world population of older adults is continuously increasing. In 1950, there were over 100 million people older than 60 years, compared to over 800 million in 2012. In the year 2050, the elderly population is expected to be greater than 2 billion people (United Nations Population Fund, 2012). Thai population is also consistent with this global trend. The proportion of older Thai people is growing rapidly along with the increased life expectancy. In 2020, Thai elderly population is predicted to be 15 percent of total population (Satiennoppagao *et al.*, 2011). Therefore, Thailand is in aging society in which the proportion of population in age over 60 years old is over 10% of all population (Statistical Forecasting Bureau, National Statistical Office, The Government Complex, 2014).

Healthy living and good nutrition are two main lifestyle factors to live longer. Since elderly people become less active with slow metabolism, have reduced appetite and poor oral health, they tend to have reduced dietary intake. Consequently, nutritional problems have risen with aging (IOM, 2010). Studies in many countries found that older adults were undernourished (Johansson, 2009) due to inadequate intakes in both macro- and micronutrients (Zhu *et al*., 2010; De Groot *et al*., 1999). Nutrient deficiencies are linked to risks of many non-communicable diseases (NCDs). For example, dietary fiber consumption has been shown to be associated with a lower risk of cardiovascular disease incidence (Mozaffarian *et al*., 2003) and reduced mortality rate of coronary heart diseas (Liu *et al*., 2003) in the elderly. Also, high fiber intake was shown to be associated with reduced colorectal cancer risks in many studies. (Levi *et al*., 2001; Gonzalez *et al*., 2004; Peters *et al.*, 2003). Additionally, vitamin B_{12} deficiency is a common cause of neuropsychiatric symptoms, such as neurologic, cognitive, psychotic, mood symptoms, and otherwise, megaloblastic anemia, gastrointestinal symptoms in elderly persons (Lachner *et al.*, 2012; Baik *et al*., 1999). Furthermore, calcium is also a nutrient of concern in elderly

population. Age-related renal impairment leads to malabsorption of calcium and accelerated bone loss (Selhub *et al*., 1995). Studies in many developing countries consistently showed that osteoporosis became a major problem associated with higher age and low-calcium diet)Katherine *et al*., 2001(. According to 2017 food consumption behavior survey, over 70% of Thai elderly consumed meat, milk and dairy products no more than once a week and only 50% of Thai elderly consumed vegetable/ fruit once a day (National Statistical Office, 2018). Nevertheless, adequacy of dietary fiber, vitamin B_{12}, and calcium intakes in Thai elderly were largely unknown. This article presented up-to-date information on adequacy of dietary fiber, vitamin B_{12}, and calcium intakes in Thai older adults. The findings from this work indicated the needs to fortify diet with certain nutrients to prevent health risks in older adults.

2 MATERIALS AND METHODS

2.1 Participants

Ninety Thai participants were recruited from the dental clinic of Golden Jubilee Medical center, Phutthamonthon Hospital, and residential area near Mahidol University, Thailand. All participants were screened based on the following inclusion criteria: being 51-79 years old; having no systemic disease or having controlled systemic diseases; having ability to eat by mouth; having Thai nationality and living in Thailand at least 5 years; being well-communicated in Thai language. Exclusion criteria were as follows: having acute inflection, neurological disorders such as stroke (symptomatic) and Alzheimer, psychosis, chronic renal failure, late-stage cancer; choke of food into the lungs (aspiration pneumonia). Before data collection, participants were informed about the aim of the study and signed their written informed consent. Their identities have been protected, following International Conference on Harmonization- Good Clinical Practice (ICH-GCP). In this study, we defined the participants aged fifty years old or above as older persons, according to a World Health Organization's statement (World Health Organization, 2002).

2.2 Sample Size Calculation

This work is a secondary data analysis from a research project "A comparative study of nutritional status and dietary intake among Thai adults with various degrees of tooth loss". The sample size in the project was calculated based on comparison between three groups. 84 samples were required to achieve power of 0.9 for effect size of 0.4 and alpha at 0.05. Adding 5% drop-out rate, 90 participants were recruited in this study.

2.3 Study Procedures

This study was approved by Mahidol University Central Institutional Review Board (MU-CIRB) with the approval number 2017/177.0910. The study was performed according to Declaration of Helsinki. This was a cross-section observational study. The data of all participants were collected at Institute of Nutrition, Mahidol University, Thailand. After passed the screening and signed the informed consent, consumption frequencies of green leaf and yellow vegetables, meat, and dairy products were assessed by food frequency questionnaire (FFQ). The amount of dietary fiber, vitamin $B_{12,}$ and calcium intakes were assessed by 4-days food record and nutrient analysis.

2.4 Outcome

The outcome of this study included amount of dietary fiber, vitamin B_{12}, calcium intakes per day, consumption frequencies of green leaf and yellow vegetable, meat, and dairy products in Thai older adults.

2.5 Consumption Frequencies of Vegetables, Meat, Milk and Dairy Products

Food frequency questionnaires (FFQ) was used to evaluate consumption of green leaf and yellow vegetables, meat, and dairy products (Jongsuwat, 2013). The choices of consumption

frequencies included 1 time/day, 2 times/day, 3 times/day, 1-2 times/weeks, 3-4 times/weeks, 5-6 times/week, and none. For statistical data analysis, the frequency categories were re-arranged as daily, weekly, and none.

2.5 Daily Intakes of Dietary Fiber, Vitamin B_{12}, and Calcium

Daily intake of dietary fiber, vitamin B_{12}, and calcium were analyzed by 4-day food record. All participants recorded the type and amount of food they consumed all day during Thursday, Friday, Saturday and Sunday of the same week. Specific brand titles of the packaged food were also included in the record. The food diaries were divided into 5-7 meals depending on each participant such as breakfast, lunch, dinner, and snacks (before breakfast, midmorning, afternoon, and late-evening snacks). Prior to self-record, a researcher taught all participants how to write the 4-day food record, provide some examples an asked the subjects to try make some records based on dietary recall. Then, all participants were asked to record their intake during 4 days by themselves. To ensure the accuracy of record, the contact information of researcher was provided in case of questions during record. After the food record had been returned, researcher checked the completeness of record and made some follow-up phone calls in case of unclear records. The amount of dietary fiber, vitamin B_{12}, and calcium intakes each day were analyzed by INMUCAL-Nutrient V.3, a software for nutrient analysis based on Thai food composition database (Ivanovitch K, *et al.*, 2014(. Average daily intakes of dietary fiber and vitamin B_{12}, and calcium were calculated by [(average of weekend days x 2) + (average of weekdays x 5)] / 7. Then, adequacy of intakes was analyzed by comparing the average daily intakes with the corresponding Thai daily recommended intake (DRI) (Committee on DRI, 2003). For calcium and vitamin B12, the average daily intakes were calculated as % RDA by dividing with the average daily dietary intake with the Thai Recommended Dietary Allowances Thai RDA (levels. Consumption of at least 70% RDA is considered adequate, while consumption of less than 70% was inadequate. Since there were no established RDA for dietary fiber, Thai recommended daily intake (RDI) of 25 g per day for Thai people aged 6 years and above was used as reference level. Since all participants consumed dietary fiber less than 70% of Thai RDI. The dietary fiber intakes were separated into three categories included 50-70% RDI (12.5-17.5 g), 20-50% RDI (5-12.5 g), and less than 20% RDI (< 5g).

2.7 Statistical Analysis

Graphing and statistical analysis were performed by using GraphPad Prism V.7.0. Characteristics of participants were presented as number and % total participants. Consumption frequencies of vegetable, meat, milk and dairy products were shown as % total participants for daily, monthly and no consumptions. Adequacy of calcium and vitamin B12 intakes were shown as % total participants for adequate and inadequate intakes. Intakes of dietary fibers were shown as % total participants for consumptions of 50-70% RDI, 20-50% RDI and less than 20% RDI.

3 RESULTS

3.1 Demographic and Health Information

Table 1 presents the demographic and health characteristics of participants. The average age of the participants was 65 years; a large majority were women (70%) and living in central of Thailand, having controlled systemic diseases (metabolic syndrome). Majority of participants graduated from primary school.

Table 1: Characteristics of the participants and proportions (%)

Characteristics	Participants (N=90)	
	n	%
Age		
Range (years)	51-78	
Age (Mean; SD)	65	6.9
Gender		
Female	63	70
Male	27	30
Living area		
Bangkok	17	19
Central excluding Bangkok	73	81
History of systemic diseases		
No disease	16	18
DM, DLP, HT (only)	10	11
DM, DLP, HT (with other)	48	53
Other diseases (bone, GI, anemia, stroke, CVD, CKD, thyroid, bladder stone, fatty live, epilepsy, asthma, allergy, migraine, BPH)	16	18
Education		
None	17	19
Primary school	35	39
Secondary school	15	17
Diploma	9	10
Undergraduate	12	13
Graduate	2	2

3.2 Inadequacy of Dietary Fiber Intake and Frequency of Vegetable Consumption

100% of participants had inadequate intake of dietary fibers (consumed less than 70% of Thai RDI). As shown in **Fig. 1**, 46% and 45% of participants consumed dietary fiber only 20-50% of RDI and 50-70% of RDI, respectively. Furthermore, 9% of participants consumed very low level of dietary fiber (< 20% of RDI). Consistently, **Fig. 2a** and **2b** showed that up to 55% of participants consumed green leaf and yellow vegetables only once a week. While up to 20% of participants did not consume vegetables at all.

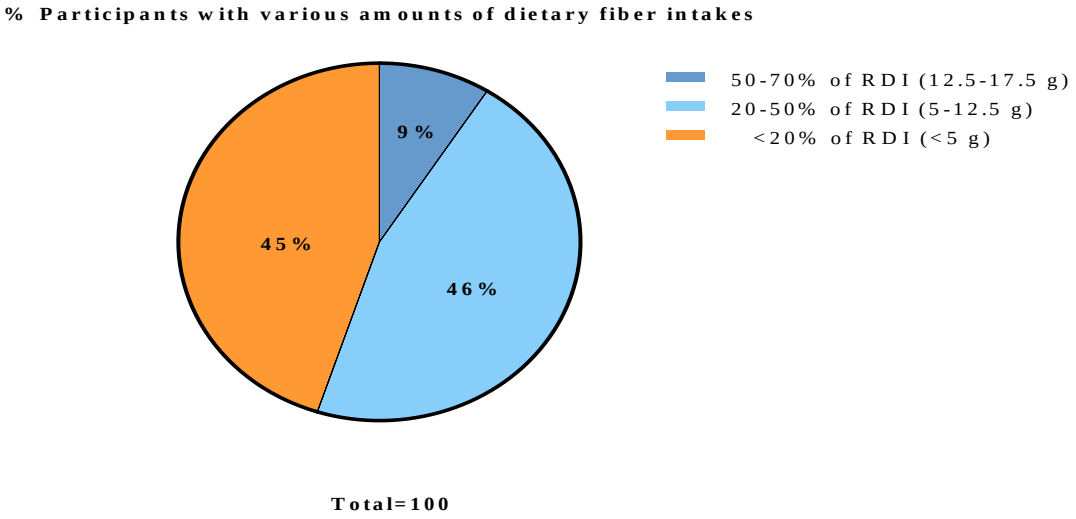


Figure 1: Adequacy of dietary fiber intake in Thai elderly
Pie chart represented the percentage of participants who had consumed dietary fiber, blue part; dietary fiber intake of 50-70% RDI (12.5-17.5 g), light blue part; dietary fiber intake of 20-50% RDI (5-12.5 g), and orange part; dietary fiber intake of < 20% RDI (< 5 g).

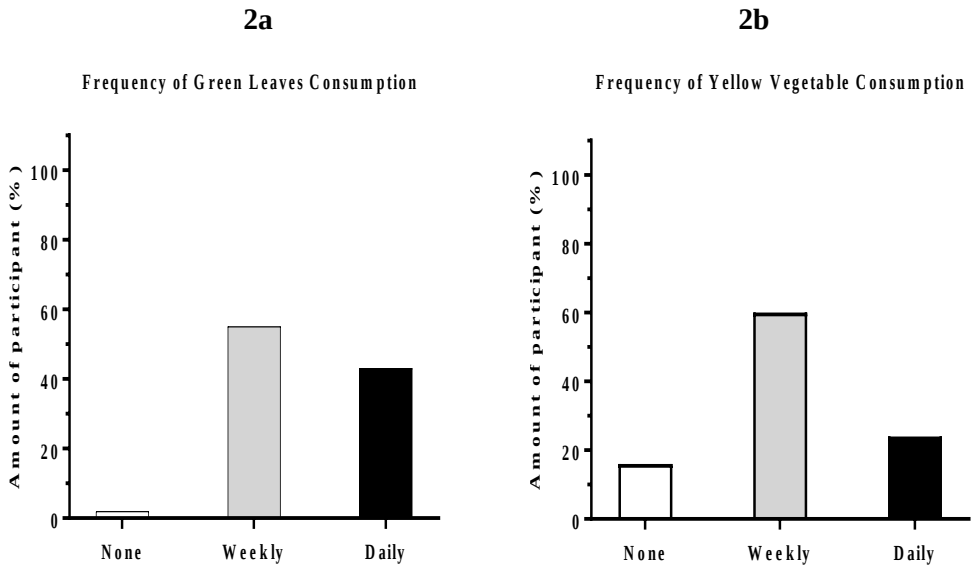


Figure 2: Frequency of green leaves (2a) and yellow (2b) vegetable consumption in Thai elderly
Each bar represented the percentage of participants who had consumed green leaves (i.e. morning glory, kale, and ivy gourd) **(2a)** or yellow vegetable (i.e. tomato, carrot, and pumpkin) (**2b**). Black bar = daily intake, gray bar= weekly intake, white bar = none.

3.3 Inadequacy of Vitamin B_{12} Intake and Frequency of Meat Consumption

As shown in **Fig 3**, there were over 70% of participants having inadequate intake of vitamin B_{12}. Consistently, almost 40% of participants consumed meat only once a week as shown in **Fig 4**. While up to 45% of participants did not consume meat at all.

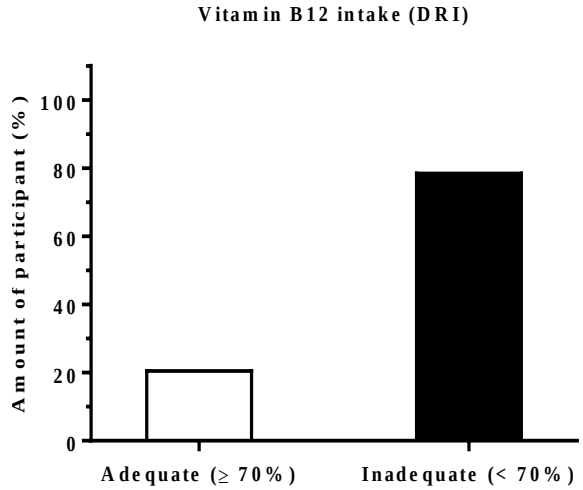


Figure 3: Adequacy of vitamin B_{12} intake in Thai elderly
Each bar represented the percentage of participants who consumed inadequate amount of vitamin B_{12} (< 70% RDA) (black bar) or adequate amount of vitamin B_{12} (≥ 70%) (White bar).

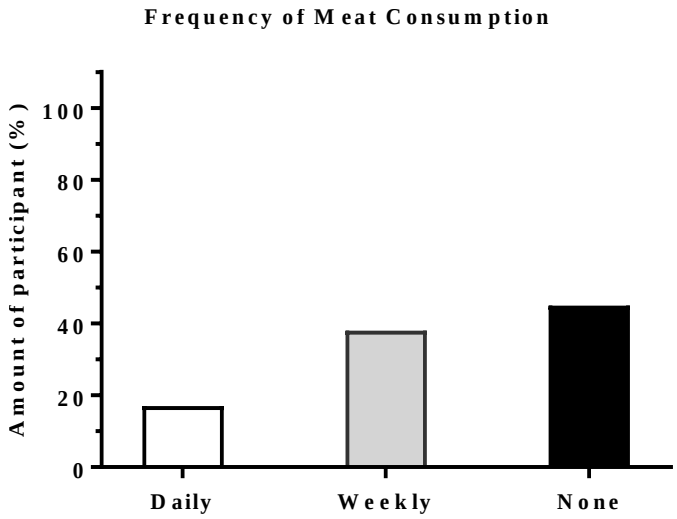


Figure 4: Frequency of meat consumption in Thai elderly
Each bar represented the percentage of participants who had consumed meat (i.e. beef/ pork/ chicken/ duck with skin)d aily (white bar), weekly (gray) or none (black bar).

3.4 Inadequacy of Calcium Intake and Milk and Dairy Product Consumption

As shown in **Fig 5**, there were 90% of participants having inadequate calcium intake (<70% DRI). Consistently, up to 30% of participants consumed milk only once a week as shown in **Fig 6**. While up to 40% did not consume milk and dairy product at all.

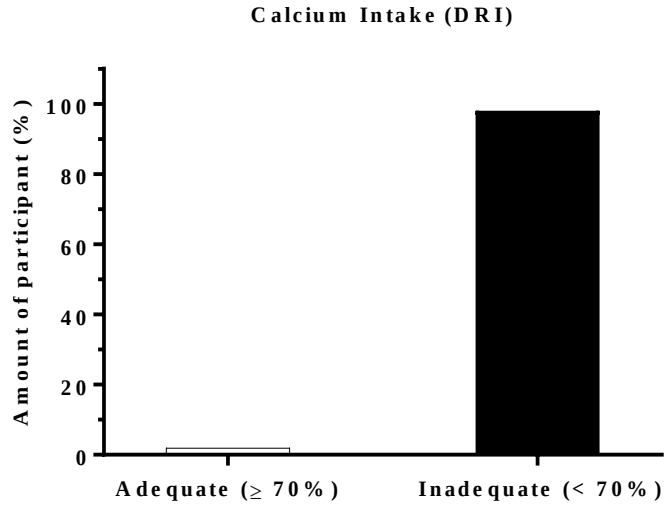


Figure 5: Adequacy of calcium intake in Thai elderly
Each bar represented the percentage of participants who consumed inadequate calcium intake (black bar; consume calcium < 70%) and adequate calcium intake (white bar; consume calcium ≥ 70%)

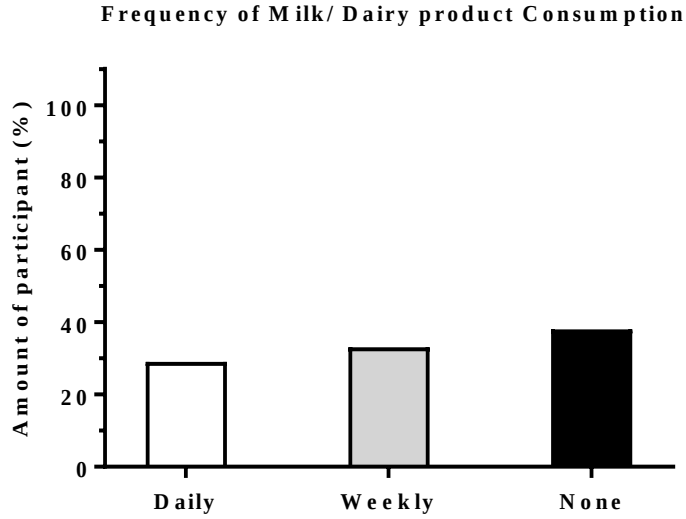


Figure 6: Frequency of milk and dairy product consumption in Thai elderly
Each bar represented the percentage of participants who had consumed milk and dairy product (i.e. fresh milk, low fat milk, unsweetened milk, and yogurt) daily (white bar), weekly (gray) or none (black bar).

4 DISCUSSION

Nutritional problems are among the most common problem in elderly population. Studies in many countries consistently showed that obesity and co-morbidities, use of medication, poor oral health, psychological, cultural or social factors can influence nutritional status in the elderly (Sanchez-Garcia, 2007; United Nations, 2005; Brownie, 2006; Moynihan, 2007). Proper and adequate nutrient intake is essential for maintaining optimum health and satisfactory functional capacity. Good nutrition could improve quality of life, prevent diseases and reduce medical expense) Bureau of Dental Health, 2012). Therefore, balanced diet may reduce risks of chronic diseases (Zhu, 2014; Bidlack, 1996). Previous studies had shown increased needs of some nutrients in older adults. For example, a previous study showed reduced bioavailability of vitamin B_{12} and calcium with increased age (Baik *et al.*, 1999, Selhub *et al*., 1995). Therefore, elder requires more vitamin B_{12} and calcium in each meal. Interestingly, calcium and vitamin B_{12} shared common food source including meat and milk. In addition, reduction

of dietary fiber intake was found in older adults associated with decreased vegetable consumption due to tooth loss (Chen, 2003). Therefore, there are needs in elders to meet those nutrients requirement. Food fortification with specific nutrients is a good approach to solve nutrient deficiency problem in a large population. In developed countries, there are many fortified products available for elderly such as cereals fortified with vitamin B_{12} and fiber, and dairy products fortified with calcium. Nevertheless, very few data are available regarding adequacy of nutrient intake in Thai elderly population. This pilot study demonstrated that most Thai older adults consumed inadequate amounts of calcium, dietary fiber and vitamin B_{12} associated with low intakes of meat, vegetables and dairy products. Therefore, fortification of these nutrients into Thai food may be in need to improve balanced nutrition and prevent chronic disease.

Previous studies expressed that micronutrients inadequacies are common among elderly. Even in the well-developed countries like United States, older adults were found to have difficulty in intestinal absorption of vitamin B_{12} due to atrophic gastritis, a degenerative stomach condition estimated to affect 25-40% of elderly population (Evans *et al.*, 1997). Likewise, a Latin America study found prevalence of vitamin B_{12} deficiency in 51% of men and 31% of women. Consistently, our current study in 2018 found that 70% of Thai elders inadequately consumed vitamin B_{12}. This is somewhat much larger than 7% vitamin B_{12} deficiency reported in 2001 study (Tucker & Buranapin, 2001). The reason underlying inadequate vitamin B_{12} intake may be associated with low meat intake. In 2017, a previous survey in Thailand found that up to 20% of Thai elderly tend to not consume meat (National Statistical Office, 2018). Interestingly, our current study in 2018 found that 40% of Thai elders did not consume meat at all. Taken together, these findings suggest a trend toward reduction of meat consumption and vitamin B_{12} intake in Thai elders. Future studies to investigate blood levels of vitamin B_{12} may be useful to characterize profile of vitamin B_{12} deficiency.

Calcium is also nutrient of particular concern for elderly populations. From a previous survey in Thailand, up to 40% of Thai elderly tended to not consume milk and dairy products (National Statistical Office, 2018. Consistently, our current study found that 40% of participants did not consume milk and dairy products at all. Milk is one of the most important source of vitamin B_{12} and calcium, which are required for the elderly to maintain bone and teeth. Thus, low intake of milk and dairy products may be an important cause of inadequate calcium and vitamin B_{12} intakes observed in Thai elders. These findings are in accordance with those in American elderly population. The largest nutrition survey in US, NHANES 2001-2002 reported high percentages of inadequate calcium intake (Moshfegh *et al.*, 2005). Similarly, another study in Arizona region found incidence of low calcium intake in more than 80% (Foote *et al.*, 2000). The observation is in line with the finding that fewer than 5% of participants consumed the recommended daily servings of dairy products. Usually, it is difficult to achieve calcium requirement without including dairy products.

The strength of the present study is the use of 4-day food record to assess nutrient intake instead of 24-hour recall commonly used in other surveys. Therefore, the amount of nutrients reported in this study is quite reliable. Nevertheless, the study had a few limitations. First, this research is a cross-section observational study. Therefore, we could not conclude causal relationship between the observed reduced nutrients intakes and food group consumption. Second, this study did not exclude participants who had nutrient supplement intake such as calcium supplement. Thus, the results may be underestimated. Future research in large-scale population should be performed with exclusion of participants using dietary supplements.

In conclusion, this study suggested that a high percentage of Thai elders consumed inadequate amounts of dietary fiber, vitamin B_{12}, and calcium intakes. Development of food product for elderly should consider fortification of these nutrients.

5 ACKNOWLEDGEMENTS

The authors are thankful to all subjects; Ms. Kanoknun Vittayakasemsont, Ms. Wassana Pookate, and Ms. Nattvara Nirdnoy for consulting, data collection and data processing.; dental staffs of Phutthamonthon Hospital and staffs of Institute of Nutrition, Mahidol University for facilitating data collection.

Funding**:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCE

- Adams, K. F., Schatzkin, A., Harris T. B., *et al*. (2006). Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old. *The New England Journal of Medicine*, 355 (8), 763–778.
- Baik, HW., Russell, RM., (1999). Vitamin B12 deficiency in the elderly. *Annual Review of Nutrition*, 19, 357-377.
- Bidlack, WR. (1996). Interrelationships of food, nutrition, diet and health: the National Association of State Universities and Land Grant Colleges White Paper. *Journal of the American College of Nutrition*, 15, 422–433
- Brownie, S. (2006). Why are the elderly individuals at risk of nutritional deficiency? *International Journal of Nursing Practice*, 12, 110–118.
- Bureau of Dental Health, Department of health. (2012). The survey report of Thailand people by oral health examination 7th. Available at http://dental.anamai.moph.go.th/survey7.pdf. Accessed on 10st August 2018.
- Committee on DRI, Bureau of Nutrition, Department of Health, Ministry of Health, Thailand, (2003). Dietary reference intake tables for Thai 2003. Bangkok: Express Transportation Organization Printing House
- De Groot, CP., van den Broek, T., van Staveren, W. (1999). Energy intake and micronutrient intake in elderly Europeans: seeking the minimum requirement in the SENECA study. *Age Ageing*, 28, 469–474.
- Evans, W. J., Cyr-Campbell, D. (1997). Nutrition, exercise, and healthy aging. *Journal of the American Dietetic Association*, 97, 632–638.
- Foote, J. A., Giuliano, A. R., Harris, R. B. (2000). Older Adults Need Guidance to Meet Nutritional Recommendations. *Journal of the American College of Nutrition*, 19(5), 628–640.
- Freedman, D. M., Ron, E., Ballard-Barbash, R., Doody, M. M., Linet, M. S. (2006). Body mass index and all-cause mortality in a nationwide US cohort. *International Journal of Obesity*, 30 (5), 822–829.
- Gaziano, JM., Manson, JE., Branch, LG., Colditz, GA., Willett, WC., Buring, JE. (1995). A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. *Annals of Epidemiology*, 5, 255–260.
- Gonzalez, CA., Navarro, C., Martinez, C., Quiros, JR., Dorronsoro, M., Barricarte, A., *et al*. (2004). The European prospective investigation about cancer and nutrition (EPIC). *Revista Espanola de Salud Publica*, 78, 167-76.
- IOM (Institute of Medicine). (2010). Providing Healthy and Safe Foods as We Age: Workshop Summary. Washington, DC: The National Academies Press.
- Ivanovitch, K., Klaewkla, J., Chongsuwat, R., Viwatwongkasem, C., Kitvorapat, W. (2014). The intake of energy and selected nutrients by thai urban sedentary workers: an evaluation of adherence to dietary recommendations. *Journal of Nutrition Metabolism,* 1-17.
- Johansson, L., Sidenvall, B., Malmberg, B., *et al*. (2009). Who will be malnourished? A prospective study of factors associated with malnutrition in older persons living at home. The *Journal of Nutrition, Health & Aging*, 13, 855–861.
- Jongsuwat, R. (2013). Food frequency questionnaire for dietary behavior: national campaign for sweet, fat, salt reduction to reduce obesity and diseases. Bureau of Nutrition, Department of Health, Ministry of Health, Thailand. http://nutrition.anamai.moph.go.th/ewt_news.php?nid=120 Accessed 18 August 2018
- Tucker, K.L., Buranapin, S. (2001) Nutrition and aging in developing countries. *Journal of Nutrition*, 131(9), 2417S-23S.
- Knekt, P., Reunanen, A., Jarvinen, R., Seppanen, R., Heliovaara, M., Aromaa, A. (1994). Antioxidant vitamin intake and coronary mortality in a longitudinal population study. *American Journal of Epidemiology*, 139, 1180–1189.

- Lachner, C., Steinle, NI., Regenold, WT. (2012). The neuropsychiatry of vitamin B12 deficiency in elderly patients. *The Journal of Neuropsychiatry and Clinical Neurosciences*, 24(1), 5-15.
- Levi, F., Pasche, C., Lucchini, F., La Vecchia, C. (2001). Dietary fibre and the risk of colorectal cancer. *European Journal of Cancer*, 37, 2091-2096.
- Liu, S., Sesso, HD., Manson, JE., Willett, WC., Buring, JE. (2003). Is intake of breakfast cereals related to total and cause-specific mortality in men?. *The American Journal of Clinical of Nutrition*, 77, 594-599.
- Malnick, S. D. H., Knobler, H. (2006). The medical complications of obesity. *An International Journal of Medicine*, 99 (9), 565–579.
- Moshfegh, A., Goldman, J., Cleveland, L. (2005). What we eat in America, NHANES 2001-2002: usual nutrient intakes from food compared to dietary reference intakes. Washington (DC): USDA Agricultural Research Service;[cited 2011 Oct 23]. Available from: http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/0102/usualintaketables2001-2.pdf
- Moynihan, P. (2007). The relationship between nutrition and systemic and oral well-being in older people. *Journal of the American Dental Association*, 138, 493–497.
- Mozaffarian, D., Kumanyika, SK., Lemaitre, RN., Olson, JL., Burke, GL., Siscovick, DS. (2003). Cereal, fruit, and vegetable fiber intake and the risk of cardiovascular disease in elderly individuals. *JAMA*, 289, 1659-1666.
- Must, A., Spadano J.,Coakley, E. H., Field, A. E., Colditz, G., Dietz, W. H. (1999). The disease burden associated with overweight and obesity. *The Journal of the American Medical Association*, 282 (16), 1523–1529.
- National Statistical Office. (2018). The 2017 food consumption behavior survey. Statistical Forecasting Division, The Government Complex, Chaeng Watthana Rd., Laksi, Bangkok, Thailand, 16-20.
- Peters, U., Sinha, R., Chatterjee, N., Subar, AF., Ziegler, RG., Kulldorff, M., *et al.* (2003). Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. The *Lancet*, 361, 1491-1495.
- Pillow, PC., Duphorne, CE., Chang, S., Contois, JH., Spitz, MR., Hursting, SD. (1999). Development of a database for assessing dietary phytoestrogen intake. *Nutrition and Cancer*, 33, 3–19.
- Sanchez-Garcia, S., Garcia-Pena, C., Duque-Lopez, MX., *et al*. (2007). Anthropometric measures and nutritional status in a health elderly population. *BMC Public Health*, 7(2).
- Satiennoppagao, W., Aekpalagorn, W., Gasemsub, R., Pakjareon, H. (2011). The survey report of Food consumption data of Thailand people (The health survey report of Thailand people by physical examination 4th in 2008-2009). Edition 1, Nonthaburi: office.
- Statistical Forecasting Bureau, National Statistical Office, The Government Complex. (2014). The 2014 survey of the older persons in Thailand. Text and Journal Publication Co., Ltd. 19.
- Trichopoulou, A., Naska, A., Vasilopoulou, E. (2001) Guidelines for the intake of vegetables and fruit: the Mediterranean approach. *International Journal for Vitamin and Nutrition Research*, 71(3),149-53.
- United Nations Population Fund, Help Age International. (2012). Aging in the twenty-first century. http://www.unfpa.org/public/op/edit/home/publications/pid/11584 Accessed 20 April 2017.
- United Nations Press Release POP/918. (2005). World population to increase by 26 billion over the next 45 years. Available at http://www.un.org/News/press/docs/2005/pop918.doc.htm Accessed on 20th August 2018.
- Watanabe, F. (2007). Vitamin B12 Sources and Bioavailability. *Experimental Biology and Medicine*, 232(10), 1266-1274.
- World Health Organization. (2002). Proposed working definition of an older person in Africa for the MDS Project. http://www.who.int/healthinfo/survey/ageingdefnolder/en/ Accessed 17 May 2017

- Zhu, K., Devine, A., Suleska, A., *et al*. (2010). Adequacy and change in nutrient and food intakes with aging in a seven year cohort study in elderly women. *The Journal of Nutrition, Health & Aging*, 14, 723–729.
- Zhu Y, Hollis JH. (2014). Tooth loss and its association with dietary intake and diet quality in American adults. J Dent, 42(11):1428-1435.

Identification of Bacteria Isolated from the Food Contact Surfaces in Poultry Processing Plant

Pawarisa Boonkoy[1], Warapa Mahakarnchanakul[2*]

[1]The Graduate School, Kasetsart University, Bangkok 10900, Thailand
[2]Department of Food Science and Technology, Agro-Industry Faculty, Kasetsart University, Bangkok 10900, Thailand
*Corresponding Author **(**fagiwpm@ku.ac.th)

Abstract

Processing equipments and environment are considered to be major concerns of microbial harborage particular in poultry processing industry. Products could potentially be susceptible to cross-contamination during processing and handling, or post contaminate after cook processing. This research focused on microbial population adhered to the equipment surfaces and environment in a poultry processing plant in Thailand. The observation was focused on 9 different locations, the samples were collected at 1, 3, 5, 7, 9 and 11 weeks. The population of microorganism adhered on the surfaces were enumerated by using swab test, bacterial count by spread plate technique and identify the unknown culture by Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF). The total plate count indicated that biofilm might form at some locations; niches, wet area or unclean surfaces, that microbial growth was over 4 log CFU/cm^2 after five weeks. The highest viable bacterial population was found in the drainage. Obtained results showed that *Achromobacter sp.* (44%) was the highest genus that found on the surface. Pseudomonadaceae (34 %), particular *Pseudomonas aeruginosa*, was found 20% of these group. Both genus is known as the potent biofilm producer. Staphylococcaceae (5%), *Staphylococcus aureus* and *Staphylococcus epidermidis* were the most frequently detected, known as as a cause of spoilage and possible to risk of foodborne illnesses. Enterobacteriaceae (3%) such as *Escherichia coli* was also identified. None of Listeria and Salmonellae were found. This study supports as a MALDI-TOF MS being a rapid tool for the identification of microbial, and present an information leading to create a cleaning program for monitoring the specified bacteria on the surfaces to control the food safety in food process.

KEYWORDS: Biofilm, Cleaning, Food Safety, MALDI-TOF, Poultry Process

1 INTRODUCTION

Microbial life abounds on surfaces in both natural and industrial environments, one of which is the food industry. The bacterial contamination of food-contact surfaces is a major factor in pathogen persistence in food processing environments, and is believed to have a significant public health impact. Indeed, even if in industrialized countries the percentage of people suffering from food-borne diseases each year has been reported to be up to 30% (WHO, 2002). Bacterial adhesion is the initial step in colonization and the formation of a biofilm on a surface. Biofilm can be occurred on a variety of food contact surfaces, the population of bacteria found in biofilm multiplies at various stages of growth and the species of microorganisms are as diverse as a number (Donlan, 2002). A significant number of the persistence of some foodborne pathogens on food contact surfaces and biofilm was reported that affects on the quality and safety of the food products (Bower *et al.*, 1996). Outbreaks of pathogens associated with biofilms have been related to the presence of *Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Salmonella* spp. *Staphylococcus* spp. and *Escherichia coli* O157:H7 (Aarnela *et al*., 2007; Waak *et al*., 2002). These contaminating microorganisms can form biofilms that are difficult to eliminate and can act as a harbour of source of contamination increasing of dissemination during food processing (Lehner *et al*., 2005; Lomander *et al*., 2004; Møretrø & Richard, 2004). The most significant species in poultry process was *Salmonella,* which were found from dust, surfaces, feces, poultry feed, and transportation of live poultry between production and processing units (Park *et al*., 2011; Ramesh *et al*., 2002). Furthermore, approximately 50% of the strains isolated on poultry farms

were able to produce biofilms (Marin *et al*., 2009).

Identification of bacteria is a systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture. It produces benefits for many aspects of the microbial research, in food industry it will help to design and implement the proper cleaning and sanitization procedure to prevent and control source of contamination (Adzitey *et al*., 2013). In this study, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed due to the rapid one step extraction method and the accuracy of identification of microorganism. Meanwhile using microbial enumeration was compared to monitor of the identified microorganisms will assist to focus on the potent biofilm forming species and control of pathogenic species (Carbonnelle *et al.*, 2011).

This study aim to investigate the population of microbial adhere on the surface at different locations and different interval of time in poultry processing plant, which will help to improve the effectiveness of cleaning process. To evaluate the cleaning process by the performance of MALDI-TOF MS for identification bacteria that may possible form and adhere on the specific food surface.

2 MATERIAL AND METHODS

2.1 Poultry Processing Plant

Samples were taken from the surface equipment, based on swabbing method at designed locations at a high risk area in the poultry processing plant at a province located at the central part of Thailand. The experiment was conducted during June to December 2017. Nine specific locations including food and non-food contact surfaces at a conveyor belt, a wire spiral belt in the freezer, after freezing at a shooter plate, a blue plastic slope belt, a transfer conveyor (prior to packaging section), floor and drain were investigated. Cleaning and sanitization program in this process plant was daily applied by high-pressure injection cleaning with alkali foam cleaner, and routinely used QACs (Quaternary ammonium compounds) as a sanitizer and PAA (Peracetic acid) was adopted once a week for sanitization.

2.2 Microbiological Analysis

Swab samples for microbiological analysis were taken from the 10x10 cm^2 surface area of assigned locations. Before performing swab test, each surface was rinsed with 10 mL sterile water, then twice swab with sterile cotton swab moisted with sterile saline. Next each swab head was broken into a plastic tube containing 10 mL of sterile physiological saline (0.85% NaCl), shaken for 1 minute. Total plate count with the spread plate technique was done by serial 10-fold dilutions for each samples and 0.1 mL dropped duplicate on Tryptic soy agar (TSA). The plates of TSA were incubated at 37 ^oC for 24 hours, counted and expressed as log CFU/cm^2. The 10 percent of isolates were chosen from each plates of TSA, then re-streaked on TSA to obtain the unknown single colony surface, then all isolates were further identified.

2.3 MALDI-TOF MS for Identification Step

MALDI-TOF MS is a mass spectrometry-based microbial fingerprinting which considered as a rapid, cost-effective, and simple method for microbial identification. The detection mainly based on the most abundant and conserved ribosomal protein fractions of bacteria resulting for classification of the organisms (Lay, 2001).

In direct transfer method, sterilized wood toothpick was used to pick a single colony, freshly "grown material" (overnight colony) should be used, then smeared as a thin film directly onto a spot on a MALDI target plate. The spots were overlayed with 1 µl of HCCA solution, left 1 hour and dried at ambient temperature before analysis by MALDI-TOF MS (Bruker Daltonik, Germany).

Increase an ability of MALDI-TOF MS to identify gram-positive species by extraction spot with formic acid (Singhal *et al*., 2015), so called extended direct method. Similarly to the direct transfer method mentioned above but firstly spots were overlayed with 1 µL of 70% formic acid, allowed to dry. *Escherichia coli* was used as the reference bacteria as a control (Panda *et al*., 2014), instrument

was calibrated according to the manufacture's instruction (Bruker Daltonik, Germany).

3 RESULTS AND DISCUSSION

3.1 The Population of Microbial on The Surface

The population of surfaces was expressed as total plate count (TPC), TPC was done at specific 9 sampling locations and observed during 11 weeks (Table 1). The observation found that bacterial adhesion in all 9 locations ranged from 3-5 log CFU/cm^2. In this study, we investigated microbial contamination on 3 groups of different surfaces; food contact surface, non-food contact surface and environment area. Slope belt and shooter plate are the risk area that chicken meat are directly contacted to the surfaces. Non-food contact surfaces includes 4 locations, although food are seldom directly contacted but we found high levels of microbial, those are roller slope belt, support slope belt, tray slope belt and guard slope. The highest population was found at the environment areas are at the edge of freezer, at a floor and drain. All investigated locations had a high probability of contaminants entering the chicken product since the microbial population exceeds the factory standard that limit not over 2 log CFU/cm^2. Following the interval sampling periods from 1 to 11 weeks we found the number of population gradually increased indicating an improper cleaning procedure. The highest number was 5.90 log CFU/cm^2 at drain area, microbial population built up at floor and drain from 4 to more than 5 log CFU/cm^{2}during 7-11 weeks. These areas were always wet and not thoroughly cleaned, the condition allowed microorganisms to adhere and grow on the surfaces. Floor and drain are at locations where although food soil or debris was not visual present, but if they were not thoroughly cleaned and sanitized the microbial adhesion may occur. These unclean surfaces become an initial step in colonization and the formation of a biofilm. Biofilm is an accumulated biomass of microorganisms and extracellular polymeric substances (EPS) on a solid surface (Bogino *et al.*, 2013). EPS will be the burden for the cleaning since it will be the barrier of sanitizing agents to expose to microorganisms underneath biofilm. Secondly when the biofilm built up to the stage of release so called dispersed phase (Petrova & Karin, 2012), the floated biofilm with the abundant microorganisms will be the source of contamination in food resulting the potential cause of spoilage or even the cause of illnesses if pathogens are present (Bridier *et al.*, 2015).

According from the previous research the number of microorganisms that can be formed as a biofilm was noted as more than 4 Log CFU/area (Gram *et al*., 2007). Hereby even if the routine cleaning and sanitizing process was carried out, but the sampling swabs taken within 11 weeks were shown that biofilm may form both on non-food contact surface and food contact, at the interval period after 5 and 7 weeks respectively. Interestingly, at the environment such edge of freezer, floor and drain, biofilm was appeared at the first week indicated immediately review of cleaning procedure and practices. Applying high pressure gun for rapid cleaning has to be restricted to prevent the aerosols formation. The mechanical cleaning by scrubbing and changing of sanitizer type may be taken into consideration. The monitoring of environment such as swabs test will assist the efficiency of cleaning and frequent cleaning and sanitizing to control and prevent the biofilm formation in food plant environments.

Table 1 Population of microorganisms as total plate count (log CFU/cm^2) at different locations of processing area

Locations	Sampling time (Week)					
	1 week	3 week	5 week	7 week	9 week	11week
Slope belt	3.20	3.25	3.22	3.10	3.11	3.13
Shooter plate	3.20	3.21	3.16	3.25	3.18	4.31
Roller of slope belt	3.26	3.24	3.10	3.18	3.19	3.30
Support of slope belt	3.28	3.07	3.18	3.25	3.35	4.34
Tray under slope belt	3.42	3.78	3.37	4.44	4.22	4.33
Guard of slope belt	3.09	3.18	3.22	3.23	3.11	4.23
Floor	4.66	4.77	4.91	5.24	5.26	5.54
Drain	4.66	4.78	4.98	4.90	4.66	5.90
Edge of freezer	4.56	4.77	4.59	4.85	4.53	5.62

3.2 Identification of Bacteria Isolates Using MALDI-TOF MS

In this study 507 of bacterial isolates from the surface equipment in processing areas were identified by MALDI-TOF mass spectrometry. The isolated bacteria were shown in Figure 1. *Achromobacter sp.* (44 %) was the highest bacterial genus that found from the surfaces. From the 240 isolates, according form the matching protein profile provided from company's data base the correct identification was 98.75 % (n= 237). *Achromobacter* is an environmental bacteria, this bacteria was reported that it has innately ability to resist many antibiotics (Swenson & Ruxana, 2014). The microbial adhered on surfaces were multi-species of microorganism. The family of Pseudomonadaceae were the second family found as 34 % (n=190). The *Pseudomonas* genus corresponds to a diverse and ecologically significant group of bacteria found in numerous natural environments. Some of them are also plant, animal, and human pathogen or responsible for food spoilage (Tryfinopoulou *et al*., 2002). *Pseudomonas* spp. have ability to adapt to various conditions and to degrade a wide range of substrates organic residues, including aromatic compounds and halogenated derivatives (Souraya *et al*., 2017). Most pseudomonads known to cause disease in humans, *P. aeruginosa* are associated with opportunistic infections in human and plant pathogen (Deep *et al*., 2011). Besides the remarkable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases (Høiby *et al*., 2010). A study by Guobjornsdottir *et al.* (2005) showed *Pseudomonas* spp. (66%) can attached to the surfaces in the shrimp factory. the processing equipment of food industries harbours a microbial ecosystem both during production and after cleaning and disinfection. The microflora is partly a reflection of the raw material used (fish) and partly a reflection of the preservation parameters used in the products (e.g. NaCl and acid). *Pseudomonas* spp. and yeasts were identified after cleaning and disinfection in all of the four production environments investigated according to the study of Guobjornsdottir, 2005. *Pseudomonas* spp. remained as the dominant microflora following cleaning and disinfection. This could indicate that these microorganisms are better in adhering to surfaces, can survive without nutrients and are more resistant to sanitizers than other microorganisms (Bagge-Ravn *et al*., 2003). *Pseudomonas* spp. are resistant to several stress factors such as disinfectants, they can adhere easily to surfaces and are excellent biofilm formers (Heydorn *et al*., 2000). More importantly, it was noted that the presence of *Pseudomonas* spp. would significantly enhance the colonization of *L. monocytogenes* on stainless steel (Guðbjörnsdóttir *et al*., 2005). Amomg the isolates there were Enterobacteriaceae strains of 6 different species; *E. coli, Klebsiella oxytoca, Providencia alcalifaciens, Serratia marcescens, Serratia ureilytica* and *Serratia liquefaciens.* The family Enterobacteriaceae is ubiquitous and includes > 210 species. Some of them are are part of the normal flora of animals and many are responsible for a broad range of human infections (Jenkins *et al.*, 2016). Among them is *Escherichia coli* (*E. coli*) is an important pathogen associated with diarrheal disease and extra intestinal infections (Steiner & Richard, 2011). Both Enterobacteriaceae and *E. coli* are widely used as indicator microorganisms to monitor the hygienic quality of different categories of foods (ICMSF, 2011).

The family Staphylococcaceae were the most gram positive found in this study, including *Staphylococcus aureus* (n=18), *Staphylococcus epidermidis* (n=7) and *Staphylococcus hominis* (n=2). There were reported that about four cases in five implant infections, staphylococci are most usually caused an infection. Two species, *S. aureus* and *S. epidermidis*, account for around two-thirds of infections (Campoccia *et al*., 2006).

Microbial identification using MALDI-TOF MS can be easily performed by direct spotting an individual colony which is picked from solid culture media using a toothpick, swab or disposable loop and placed on a MALDI target slide for analysis. However, the reliably identify bacteria to species level compared to a typical cultural morphology identification particular an "uncommon" isolates may appear due to the deficiencies in the current database. An impact of growth conditions on microbial identification may include since this analysis of protein spectra based on the peptide mass fingerprint throughout mass ranges, the changes of protein profile might be relevant for microbial identification (Valentine *et al*., 2005; Walker *et al*., 2002). The microbes with a more complex cell wall will be applied a simple formic acid-acetonitrile extraction. The added extraction step is often used for in which formic acid is used for cell lysis and acetonitrile treatment precipitates protein in the sample (Anderson *et al.*, 2012).

In meat industry the crucial pathogens such as *Listeria* spp. and Salmonellae particular in the ready to eat foods is the high concern (Sofos & Ifigenia, 2010), so as to avoid of recall products the monitoring of cleaning may monitor by using the MALDI-TOF MS. The benefit of this analysis may overcome the time constraints of the output from the conventional food microbiological laboratory.

Table 2 Performance of the MALDI-TOF MS testing of bacterial identification from poultry process facilities.

Oraganism group	Total number of isolated	No. of bacteria found on the surfaces		
		Contact	Non-contact	Environment
Actinomycetaceae	**2**			
Actinomyces oris	2			2
Aeromonadaceae	**4**			
Aeromonas hydrophila	4		1	3
Alcaligenaceae	**244**			
Achromobacter sp.	240	42	53	145
Achromobacter xylosoxidans	4	1	1	2
Comamonadaceae	**11**			
Comamonas testosteroni	11	3	2	6
Corynebacteriaceae	**18**			
Corynebacterium striatum	8	2		6
Corynebacterium simulans	10	1	4	5
Enterobacteriaceae	**14**			
Escherichia coli	1		1	
Klebsiella oxytoca	2		2	
Providencia alcalifaciens	1	1		
Serratia marcescens	5	2		3
Serratia ureilytica	3	1	1	1
Serratia liquefaciens	2		2	
Flavobacteriaceae	**2**			
Elizabethkingia miricola	2			2
Micrococcaceae	**2**			
Kocuria palustris	2		2	
Moraxellaceae	**7**			
Acinetobacter haemolyticus	2			2
Acinetobacter junii	2		1	1
Acinetobacter parvus	3		1	2
Promicromonosporaceae	**12**			
Cellulosimicrobium cellulans	12	2	3	7
Pseudomonadaceae	**190**			
Pseudomonas aeruginosa	71	5	19	47
Pseudomonas rhodesiae	1			1
Pseudomonas azotoformans	1			1
Pseudomonas jessenii	1			1
Pseudomonas brassicacearum	11		3	8
Pseudomonas pseudoalcaligenes	10	1	3	6

Pseudomonas oleovorans	13		2	11
Pseudomonas chlororaphis	20	2	5	13
Pseudomonas corrugata	48	4	3	41
Pseudomonas kilonensis	4			4
Pseudomonas panipatensis	1	1		
Pseudomonas mendocina	9		3	6
Saccharomycetaceae	**1**			
Candida metapsilosis	1			1
Shewanellaceae	**12**			
Shewanella putrefaciens	12		2	10
Staphylococcaceae	**27**			
Staphylococcus hominis	2			2
Staphylococcus epidermidis	7	3	1	3
Staphylococcus aureus	18	4	3	11
Streptococcaceae	**16**			
Streptococcus pneumoniae	9	1	1	7
Streptococcus sanguinis	3			3
Streptococcus mitis	2	1		1
Streptococcus oralis	1			1
Streptococcus cristatus	1			1
Xanthomonadaceae	**8**			
Stenotrophomonas maltophilia	8	2	1	5

Table 3 Performance of the MALDI-TOF MS testing of bacterial identification from poultry process facilities by gram stain.

Families	The number of bacteria isolated	
	Gram positive	Gram negative
Actinomycetaceae	2	
Aeromonadaceae		4
Alcaligenaceae		244
Comamonadaceae		11
Corynebacteriaceae	18	
Enterobacteriaceae		14
Flavobacteriaceae		2
Micrococcaceae	2	
Moraxellaceae		7
Promicromonosporaceae	12	
Pseudomonadaceae		190
Shewanellaceae		12
Staphylococcaceae	27	
Streptococcaceae	16	
Xanthomonadaceae		8

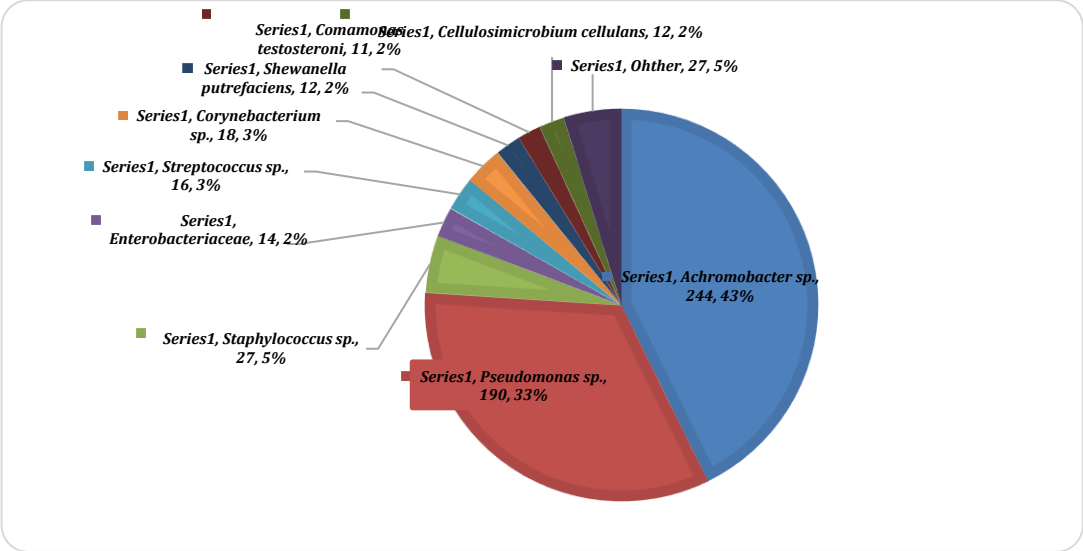


Figure 1: The ratio as percent of bacteria isolated from all 9 locations from poultry process plant

4 CONCLUSION

Microbial adhesion to surfaces and the consequent biofilm formation was presented in 9 different environments in these poultry facilities including food contact, non food and environment areas. The population of microorganism adhesion on surfaces in poultry processing plant demonstrated that inappropriate cleaning process leads to the increasing of microbial. Therefore, the prevention of distributed microorganism from biofilm in processing surfaces or environmental needed to be strictly control to prevent the spoilage of food product, and may lead to the problem of food safety. The

implementation of sanitation monitoring by adopting or considering bacterial identification by MALDI-TOF MS will assist to prevent the build up of target spoilage such as *Pseudomonas* spp. or pathogenic bacteria Staphylococcus spp. The ease and simplicity of this method will improve laboratory workflow and allow for fast corrective action for sanitation control.

5 ACKNOWLEDGEMENTS

The authors would like to thank the Faculty of Agro-Industry, Kasetsart University, Thailand. This research was financial supported by the poultry processing industry in Thailand.

REFERENCES

- Aarnela, K., Janne, L., Hannu, K. & Gun, W. (2007). Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT – Food Science and Technology*, 40, 1041–1048.
- Adzitey, F., Nurul, H. & Gulam, R. R. A. (2013). Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *Biotech*, 3, 97–107.
- Anderson, N. W., Blake, W. B., Katherine, M. R., Lauren, N. P., Stacy, G. & Nathan, A. L. (2012). Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization–time of flight mass spectrometry. *Journal Clinic Microbiol*, 50, 1008–1013.
- Bagge-Ravn, D., Yin, N., Mette, H., Jesper, N. C., Charlotte, J. & Lone, G. (2003). The microbial ecology of processing equipment in different fish industries-analysis of the microflora during processing and following cleaning and disinfection. *International Journal of Food Microbiology*, 87(3), 239-250.
- Bogino, P. C., Maria, l. M. O., Fernando, G. S. & Walter, G. (2013). The Role of Bacterial Biofilms and Surface Components in Plant-Bacterial Associations. *International Journal of Molecular Sciences*, 14, 15838-15859.
- Bower, C. K., McGuire, J. & Daeschel, M. A. (1996). The adhesion and detachment of bacteria and spores on food- contact surfaces. *Trends in Food Science and Technology*, 7,152-157.
- Bridier, A., Vizuete, P. S., Guilbaud, M., Piard, J.-C., Naïtali, M. & Briandet, R. (2015). Biofilm-
  associated persistence of food-borne pathogens. *Food Microbiology*, 45, 167-178.
- Campoccia, D, Lucio, M. & Carla, R. A. (2006). The significance of infection related to orthopedic devices and issues of antibiotic resistance. *Biomaterials*, 27, 2331-2339.
- Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin B., Beretti, J.-L., Ferroni, A., Gutmann, L. & Nassif, X. (2011). MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clinical Biochemistry*, 44, 104-109.
- Deep, A., Uma, C. & Varsha, G. (2011). Quorum sensing and bacterial pathogenicity: from molecules to disease. *Journal of Laboratory Physicians*, 3(1), 4–11.
- Donlan, R. M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases PERSPECTIVE*, 8(9), 881-890.
- Gram, L., Dorthe, B. –R., Yoke, Y. N., Pernille, G. & Birte, F. V. (2007). Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. *Food Control,* 18 (10), 1165-1171.
- Guobjornsdottir, B., Hjörleifur, E. & Guðjón**,** T. (2005). Microbial adhesion to processing lines for fish fillets and cooked shrimp: influence of stainless steel surface finish and presence of gram- negative bacteria on the attachment of *Listeria monocytogenes*. *Food Technology and Biotechnology*, 43(1), 55-61.
- Heydorn, A., Alex, T. N., Morten, H., Claus, S., Michel, G., Bjarne, K. E. & Soren, M. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*, 146, 2395–2407.

- Høiby, N., Oana, C. & Thomas, B. (2010). *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiology*, 5(11), 1663–1674.
- ICMSF (International Commission on Microbiological Specifications for Food). (2011). Microorganisms in foods 8 – Use of data for assessing process control and product acceptance. New York: Springer.
- Jenkins, C., Rentenaar, R. J., Landraud, L. & Brisse, S. (2016). Enterobacteriaceae. In Cohen, J., William, P. & Steven, O. (Eds.). *Infectious diseases* (pp. 1565–1578). (4th Eds.). Amsterdam: Elsevier Health Sciences.
- Lay, J. O. (2001). MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews*, 20 (4), 172-194.
- Lehner, A., Kathrine, R., Leo, E., Pieter, B., Benjamin, D. & Roger, S. (2005). Biofilm formation, extracellular polysaccharide production, and cell-to-cell signalling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. *Journal of Food Protection*, 68, 2287–2294.
- Lomander, A., Paul, S., Estelle, R. -C. & Laila, A. (2004). Evaluation of chlorines impact on biofilms on scratched stainless steel surfaces. *Bioresources Technology*, 94, 275–283.
- Marin, C., Hernandiz, A. & Lainez, M. (2009). Biofilm development capacity of *Salmonella* strains isolated in poultry risk factors and their resistance against disinfectants. *Poultry Science*, 88(2), 424-431.
- Møretrø, T. & Solveig, L. (2004). *Listeria monocytogenes*: biofilm formation and persistence in food processing environments. *Biofilms*, 1, 107–121.
- Panda, A., Anup, K. G., Bijey, R. M., Immaculata, X., Saikat, P., Jyotish, C. S., Alagiri, S., Shehla, K., Neha, R. & Yubhisha, D. (2014). MALDI-TOF mass spectrometry for rapid identification of clinical fungal isolates based on ribosomal protein biomarkers. *Journal of Microbiological Methods*, 109, 93–105.
- Park, S. H., Jarquin, R., Hanning, I., Almeida, G. & Ricke, S. C. (2011). Detection of *Salmonella* spp. survival and virulence in poultry feed by targeting the hilA gene. *Journal of Applied Microbiology*, 111(2), 426-432.
- Petrova, O. E. & Karin, S. (2012). Sticky Situations: Key Components That Control Bacterial Surface Attachment. *Journal of Bacteriology*, 12, 2413–2425.
- Ramesh, N., Joseph, S. W., Carr, L. E., Douglass, L. W. & Wheaton, F. W. (2002). Evaluation ofchemical disinfectants for the elimination of Salmonella biofilms from poultry transport containers. *Poultry Science*, 81(6), 904-910.
- Singhal, N., Manish, K., Pawan, K. K. & Jugsharan, S. V. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology*, 6, 791-806.
- Sofos, J. N. & Ifigenia, G. (2010). Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Science*, 86(1), 2-14.
- Souraya, B. T., Nadia, Z. J., Khelifa, B., Rayda, B. A., Hatem, R., Bilal, Z., Sidali, K., Hadjira, B., Nasserdine, S., Samir, B., Mouhamed, E. H., Abdelmalek, B., Rachid, A. & Bassem, J. (2017). Biochemical and molecular characterization of a novel metalloprotease from *Pseudomonas fluorescens* strain TBS09. *International Journal of Biological Macromolecules*, 107, 2351-2363.
- Steiner, T. S. & Richard, L. G. (2011). *Escherichia col*i enteric infections. In L. Goldman, & A. I. Schafer (Eds.). *Goldman's Cecil medicine* (pp. 1871–1873). (24th Eds.). Amsterdam: Elsevier Health Sciences.
- Swenson, C. E. & Ruxana, T. S. (2014). *Achromobacter* Respiratory Infections. *Focused Reviews*, 2, 252-258.
- Tryfinopoulou, P., Effie, T. & George, -J. E. N. (2002). Characterization of Pseudomonas spp. Associated with Spoilage of Gilt-Head Sea Bream Stored under Various Conditions. *Applied and Environmental Microbiology*, 68(1), 65-72.

- Valentine N., Sharon, W., David, W., Catherine, P. & Karen, W. (2005). Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. *Applied and Environmental Microbiology*, 71, 58-64.
- Waak, E., Wilhelm, T. & Marie, -L. D. T. (2002). Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm tanks and in dairy plant receiving tanks. *Applied and Environmental Microbiology*, 68, 3366–3370.
- Walker, J., Fox, A.J., Edwards, -J. V. & Gordon, D. B. (2002). Intact cell mass spectrometry (ICMS) used to type methicillin-resistant *Staphylococcus aureus*: media effects and interlaboratory reproducibility. *Journal Microbiology Methods*, 48, 117-126.
- WHO. (2002). Fact Sheet N 237, Food Safety and Foodborne Illness. *World Health Organization*.

Impact Assessment of Fiber Rich Supplement on Blood Glucose Level of Diabetic Subjects

Seema Medhe[1*], Asha Arya[2], Mayuri Choudhary[3]

[1]Asian Institute of Technology, Khlong Luang, Thailand
[2]Vasantrao Naik Krushi Vidyapith, Parbhani, India
[3]SDMVM's college of Food Technology, Aurangabad, India
*Corresponding Author (st116023@ait.ac.th)

ABSTRACT

An investigation was undertaken to assess the effect of the fiber-rich supplement on blood glucose level of type 2 diabetic patients. A fiber-rich supplement was tested on eleven type 2 diabetic subjects for blood glucose lowering effect by undertaking an intervention of two months feeding trials. Results for two months supplementation indicated that the fiber-rich supplement was helpful in significantly reducing the fasting and postprandial blood glucose level of subjects. The reduction in the fasting blood glucose level after two months supplement intake was 30-70 mg dL^{-1}. The postprandial blood glucose values of the subjects were reduced by 92-140 mg dL^{-1}. There was a reduction in the physiological symptoms at the end of the supplementation. It can be concluded from the study that the fiber-rich supplement had therapeutic value as it was beneficial for lowering the blood glucose content of type 2 diabetics.

KEYWORDS: Fiber Supplement, Type 2 Diabetes, Medicinal food, Glycemic index, Therapeutic food

1 INTRODUCTION

Diabetes mellitus places a very significant burden on individuals, healthcare systems, and societies in all countries. Diabetes mellitus is a chronic metabolic disorder that prevents the body to utilize glucose entirely or partially genetic predisposition combined with lifestyle changes, associated with urbanization and globalization, contribute to this rapid rise of diabetes in India (Mohan *et al.*, 2007). Diet of diabetic person should have more amount of dietary fiber as it plays a vital role in the health status of a diabetic person. Dietary fiber is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Physical properties of dietary fiber are water holding capacity (WHC), cation binding capacity (CBC) and viscosity, whereas, physiochemical properties include hypoglycemic and hypolipidemic effects. Dietary fiber promotes beneficial physiological effects including laxation and/or blood attenuation, (Hipsley, 1953). Dietary fiber and complex carbohydrates benefit Type 1 and Type 2 diabetes. Soluble dietary fiber such as pectin, gums, hemicelluloses increase intestinal transit time, delay gastric emptying, slow glucose absorption and lower serum cholesterol (Shrilaxmi, 2006).

Soluble dietary fiber binds a large quantity of water and becomes viscous in the stomach. Thereby it delays the secretions of the gastrointestinal tract, which results in reduced digestion of food material and finally delaying the absorption of glucose. Due to delayed absorption of glucose, secretion of insulin by pancreas will also be delayed. Such condition results in activation of the receptors of the cells in target organs resulting in increased insulin sensitivity for glucose uptake by the cells. Hence, there will be a reduction in the blood glucose level. Plant foods are the only sources of dietary fiber. Vegetables (particularly leafy), fruits, condiments, spices and unrefined whole grains are rich in fiber. Foods such as various gums viz. gum karaya, gum acacia, guar gum, fenugreek seeds and supplements such as bran and psyllium are rich sources of dietary fiber. The suggested intake of total dietary fiber for a diabetic person is 25 g 1000^{-1} kcal energy (Raghuram *et al.*, 2006). Many references support that the fiber supplement influences the glucose homeostasis and lower the risk of diabetes. Increased

dietary fiber intake may reduce appetite and lower total energy intake, thus reducing the adiposity and improving insulin sensitivity. Therefore, keeping in mind the benefits of fiber supplement the present investigation was undertaken for the benefits of fiber supplement to diabetic patients; its impact was also assessed.

2 MATERIALS AND METHODS

2.1 Selection of Subjects

An eminent local physician was contacted to whom the persons suffering from diabetes mellitus were visiting for treatment and follow-up regularly. After discussion with the physician regarding the fiber-rich supplement and intervention programme, a one day camp was organized for diabetic patients. For the awareness of the participants, informative talks of the nutritionists were arranged. 11 non-insulin dependent diabetic patients were selected for supplementation, which comprised of five male and six females. Subjects free from added risk factors like hypertension, thyroid, kidney disease, smoking, alcoholism and chewing tobacco were selected.

2.2 Data Collection

The two anthropometric measurements of weight and height were recorded, and BMI was calculated. Recent pathological reports of blood glucose levels of the selected subjects were examined to confirm the history of diabetes mellitus. Before starting the intervention programme, initial fasting and postprandial blood glucose levels were assessed by using Glucometer (Smart care mini make). Data regarding the baseline information of selected diabetic subjects given in Table 1.

Table 1: Baseline characteristics of the selected diabetic subjects (n=11)

Parameters	Female (mean)	Male (mean)
No. of patients	5	6
Age (years)	48.6	58.9
Duration of diabetes (years)	4	4.2
Body weight (kg)	53.9	60.67
Height (cm)	155.7	162.3
BMI	22.03	22.46
Oral hypoglycemic drug	No	No

2.3 Supplementation

Daily 40 g of the fiber-rich supplement was given to subjects divided into two equal amounts. A supplement was provided immediately after breakfast (20 g) and lunch (20 g) by adding it in water. Their daily diet was not altered throughout the study period except for the inclusion of fiber-rich supplement. The period of intervention was 60 days. The purpose of giving the supplement immediately after the meal was to fetch the benefit of the property of fiber that it delays the emptying of the stomach and helps for slow releasing of glucose in the blood. The fiber-rich supplement was having a glycemic index of 38.14 and contents 58.65g fiber.

2.4 Estimation of Blood Glucose

The blood glucose level of the selected subjects was estimated one day before the supplementation, and at the interval of 15 days till the end of the supplementation. The fasting blood glucose was determined early in the morning before the ingestion of any food material and postprandial blood level was estimated 2 hours after lunch.

2.5 Physiological Symptoms

The data on physiological symptoms experienced by selected subjects was gathered by asking them about the frequency of symptoms such as polydypsia, polyphagia, polyuria, and nocturia in 24 hours. The rate of the symptoms was noted before and after completion of supplementation.

2.6 Statistical Analysis

The data was compiled and analyzed statistically for interpretation of results. The information on blood glucose response was investigated by applying an analysis of variance, and the 'F' values were calculated to find out the difference in the blood glucose level initially and at the end of the supplementation (Panse and Sukhatme, 1985).

3 RESULTS AND DISCUSSION

The data regarding the nutrient content of selected fiber-rich supplement presented in Table 2. It can be noticed from the findings that the chosen variation was containing a high amount of total dietary fiber (58.65g $100g^{-1}$) followed by carbohydrate (20.70g). The moisture content of fiber-rich supplement was very low (2.52%). The values for protein and fat content were 6.53% and 5.02%, respectively. Total minerals were 6.2 %. The soluble fiber was 38 g, and insoluble fiber was 20-65 g. Iron and zinc contents were 0.28 mg and 0.09 mg respectively. The calculated values of calcium, thiamin, carotene, riboflavin, niacin and vitamin C were 507mg, 0.18 mg, 121.36 µg, 0.44 mg, 0.79 mg and 3.2 mg, respectively. The cost of the developed supplement was Rs. 24.44 $100g^{-1}$ which was far below the commercially available supplements.

Table 2: Nutritional composition of selected fiber rich supplement (per 100g)

Nutrient	Amount
Moisture (g)	2.52
Protein (g)	6.53
Fat (g)	5.02
Fiber (g)	58.65
Carbohydrate (g)	20.70
Iron (mg)	0.28
*Calcium (mg)	507
*Carotene (µg)	121.36
*Thiamin (mg)	0.18
*Riboflavin (mg)	0.44
*Niacin (mg)	0.79
*Vitamin C (mg)	1.1
Cost (Rs.)	24.44

The impact of fiber rich supplement on the fasting blood glucose content of the selected diabetic subjects is presented in Table 3. The initial fasting blood glucose of the subjects ranged from 120 to 178 mg dL^{-1}. There was a subsequent reduction in the fasting blood glucose of all the subjects as the period of fiber-rich supplement intake increased from the beginning of the experiment to 60 days with few exceptions. The statistical analysis of the data indicated that there was a significant reduction in the fasting blood glucose levels from the within two months after starting the consumption of fiber-rich supplement. There was 30 to 70 mg dL^{-1} reduction in the blood glucose content due to continuous consumption of fiber-rich supplement. The fasting blood glucose content was reduced to 88 to 140 mg dL^{-1} at the end of the supplementation. The fiber-rich supplement had very high fiber content (58.65%) and very low glycemic index. Hence, it might have exerted the positive effect on the blood glucose content of the diabetic subjects. The data regarding the impact of developed supplement on the post-meal blood sugar level of selected subjects depicted in Table 3.

The fasting blood glucose of selected subjects ranged from 120 to 178 mg dL^{-1} with an average blood glucose of 153.73 mg dL^{-1} which is indicative of the presence of hyperglycemia. After intake of the fiber-rich supplement by the subjects, the blood glucose content started reducing after one month which continued till the end of supplementation. At the end of the experiment, the reduction in the blood glucose content was significant, which dropped down to 88 to 140 mg dL^{-1}. The average decrease in the fasting blood glucose level was 37.46 mg dL^{-1}. After the intervention, the maximum blood glucose content was 229 mg dL^{-1} . The mean initial blood glucose content of female subjects was 148.20 mg dL^{-1} with a range of 120 to 177 mg dL^{-1} . Due to the 60 days supplementation, the fasting blood glucose was reduced to 88 to 125 mg dL^{-1} with a mean value of 108 mg dL^{-1} at the end of the experiment. The blood glucose values of the male subjects indicated that the initial fasting blood glucose ranged from 134 to 178 mg dL^{-1} with an average of 158.33 mg dL^{-1} . Which is very high than the normal value (120 mg dL^{-1}). After receiving the supplementation for two months, blood glucose values reduced to a range of 92 to 140 mg/dl with a mean decrease up to 120.67 mg/dl. The statistical difference between initial and final blood glucose values was not significant.

Table 3: Effect of fiber rich supplement on fasting blood glucose levels of selected diabetic subjects (mg dL^{-1})

Subjects	Initial	15 days	30 days	45 days	60 days
1	134	121	132	107	92
2	178	168	156	156	140
3	177	135	128	124	121
4	130	127	128	122	111
5	120	118	105	90	88
6	141	132	131	124	95
7	173	186	168	142	140
8	141	115	120	121	119
9	177	168	163	128	122
10	178	198	183	148	139
11	142	130	123	120	112
Mean	153.73	145.27	139.73	125.64	116.27
F value-3.90**		SE ± 7.49		CD-20.74	

NS- Non significant *Significant at 5 percent **Significant at 1 percent

The blood glucose values of the diabetic patients indicated that the initial postprandial blood glucose levels of the patients were very high which ranged from 190 to 295 mg dL^{-1}. As the patients started taking the fiber-rich supplement, the blood glucose values of the subjects began declining after 15 days. The reduction in blood glucose level was continued till the end of the experiment, i.e., up to 60 days. After 60 days period, the blood glucose of the patients ranged between 143 to 234 mg dL^{-1}, which indicated that there was a reduction in the blood glucose content by 24 to 73 mg dL^{-1}.

The mean postprandial blood glucose value of selected diabetic patients before starting of supplementation was 233.27 mg dL^{-1}. It was significantly reduced after supplementation (186.91 mg dL^{-1}). The maximum initial postprandial glucose level was 295 mg dL^{-1}. The postprandial plasma blood glucose values of female subjects ranged from 190 to 295 mg dL^{-1}at the beginning of the experiment. Two months of supplementation was beneficial two bring down the blood values up to a range of 143 to 234 mg dL^{-1} with an average reduction of 54 mg dL^{-1}. The postprandial blood glucose values of selected male subject initially were 190 to 274 mg dL^{-1}. The final postprandial blood glucose level ranged from 144 to 229 mg dL^{-1}. The mean initial postprandial blood glucose was 228.33 mg dL^{-1}which was reduced to 187 mg dL^{-1} due to supplementation.

Table 4: Effect of fiber rich supplement on postprandial blood glucose levels of selected diabetic subjects (mg dL^{-1})

Subjects	Initial	15 days	30 days	45 days	60 days
1	199	188	174	152	144
2	274	264	244	230	229
3	258	243	249	236	234
4	295	280	257	246	231
5	256	241	273	210	183
6	190	192	177	155	143
7	293	278	268	232	230
8	190	168	176	165	160
9	235	274	224	203	188
10	249	274	232	217	208
11	223	228	209	210	195
Mean	242	239.09	225.73	205.09	195
F value-2.80*		SE±12.05		CD-33.35	

NS- Non significant *Significant at 5 percent **Significant at 1 percent

The data for physiological symptoms of selected diabetics presented in Table 5. The mean frequency of the polydipsia, polyphagia, polyuria, and nocturia were 7.5, 4, 6.19 and 3.08 respectively before starting the supplementation. The respective frequencies after supplementation were 6.49, 3, 4.61 and 2.85 for below-referred symptoms. It can be inferred that occurrence of the signs in the initial phase of the experiment was more frequent than that of the final stage. Haripriya and Pramakumari (2010) studied the changes in physiological symptoms of diabetic subjects. They observed that polyuria, polydipsia, polyphagia, nocturia, and constipation were most commonly occurring symptoms in people with diabetes. The bran supplementation fed to the patients for six months were helped in a drastic reduction in the physiological symptoms.

Table 5: Effect of supplementation on physiological symptoms of selected subjects (n =11)

Symptoms	Initial frequency	Final frequency
Polydypsia	7.35	6.49
Polyphagia	4.0	3.0
Polyurea	6.19	4.61
Nocturea	3.08	2.85

Apart from the physiological symptoms, the data for opinions of the patients regarding the supplement and intervention programme were collected which revealed that the flatulence was experienced by two subjects, in the beginning, two-three days, afterward, it was subsided. Stomach heaviness and hardness were reduced after supplementation. The reduction in the physiological symptoms polydypsia, polyphagia, polyurea, nocturia and constipation was noticed after supplementation.

4 CONCLUSION

From the investigation, it can be concluded that locally available foods can develop fiber-rich supplement having therapeutic value. However, the developed supplement was very rich in total and soluble dietary fiber. Also, it had a very low glycemic index. Besides, the two months intervention with fiber-rich supplementation was beneficial in reducing the fasting and postprandial blood glucose levels of diabetic subjects. Moreover, the fiber-rich supplement was beneficial in reducing the physiological symptoms.

REFERENCES

- AACC Report (2001). The Definition of Dietary Fiber Cereal Food Word, Vol. 46, No.3.
- Anderson JW, Gustafson NJ, Bryant CA and Tietyan-Clerk J (1987). Dietary Fiber and Diabetes: A Comprehensive Review and Practical Application. *J. Am. Diet. Assoc*. 87.(9): 1189-1197.
- Barclay Alan W, Paul Mitchell, Victoria M, Flood, Jennie C. BrandMiller and Elena Rochtchina (2007). Glycemic Index, Dietary Fiber and Risk of Type 2 Diabetes in Cohort of Older Australians. *Diabetes Care*, Vol. 30., No. 11.
- Chandalia Manisha, Garg Abhimanyu, Lutijohann Dieter, Bergmann Kalus Von, Grrundy Scott M. and Brinkley Linda J. (2000). Beneficial Effects Of High Dietary Fiber Intake In Patients With Type 2 Diabetes Mellitus. *N. Engl.J. Med*. 342:1392- 8.
- Deshpande KB (1987). A Study on The Effects of Bran's of Different Cereals, Hulls of Different Pulses and Fenugreek Seeds on Blood Glucose Response in Normal and Diabetic Subjects. Thesis (M.Sc.) Marathwada Agriculture University Parbhani.
- Geeta C and Easwaran PP, (1990). Hypoglycemic Effects Of Millet Incorporated Breakfast Items On Selected Non-Insulin Dependent Diabetic Patients. *The. Ind. J. Jutr. Dieter*. (27): 316- 320.
- Hipsley EH, (1953). Dietary "Fiber" and Pregnancy Toxemia. *British Medical Journal* 2:420-422.
- Mani Indirani and Mani Uliyar V, (1987) Effect of Wheat Bran Supplementation On Blood Sugar, Glycosilated Protein and Seru Lipids In NIDDM Subjects. *Plant Foods for Human Nutrition* 37:161-168.
- Mani UV, Mani IU, Iyer UM, Prakash B, Manivannan T, Campbell S and Chandalia S (1997). Glycemic and Lipemic Response to Various Regional Meals and South Indian Snacks. *Int. J. Diab. Dev. Countries*, Vol. 17
- Mohan V, Sandeep S, Deepa R, Shah B and Vargahese C (2007). Epidemiology of Type 2 Diabetes: Indian Scenario. *Indian .J. Med. Res*. (125) Pp. 217-230.
- Panse VG and Sikhatme PV, (1985). Statistical Methods for Agricultural Workers. ICAR Publication, New-Delhi 58-60, 97-110.
- Raghuram T.C., Prsricha S. and Sharma R.D. (2006). Diet and Diabetes, NIN.
- Shrilaxmi B, (2006). Food Science Third Edition New Age International (P) Limited, Publishers, New-Delhi.
- Torangatti G and Naik RK (1999). Glycemic and Lipemic Response of Composite Foods In Normal and NIDDM Subjects. Int.J. Diab. Dev. Countries., Vol.19

ANNEXES

Co-funded by the
Erasmus+ Programme
of the European Union

The 1st International Conference on "Innovative Food Ingredients and Food Safety"

12-13 September 2018
Windsor Hotel, Bangkok, Thailand

The aim of this International Conference is to focus on timely and trending topics that will address the opportunities and challenges in the emerging innovative technology for sustainable and safer production of food and beverage and to bring together researchers, industries and policy developers to discuss on challenges, trends and innovative approaches for safer and sustainable food and beverage produce. The 1st day is dedicated to disseminate outcomes of 2 ERASMUS+ capacity building projects (SEA-ABT and ASIFOOD), which are related to food quality and safety of food and beverages and to a hot topic on sugar and sweeteners. The 2nd day will focus then on research results.
The following topics, but not limited will be highlighted during this conference.

ANNEX-1: CONFERENCE TOPICS

- Innovative food ingredients and food quality
- Traditional foods and beverages
- Functional foods, functional beverages and nutraceuticals
- Food safety and risk assessment analysis in food production and food supply chain systems
- Emerging trends and public health concerns of use of chemicals (Antibacterial compounds, pesticides, sterodis etc.) in food systems
- Medical foods
- Exploration and biotransformation of bioresources for high value food ingredients
- Application of biotechnology and nanotechnology in sustainable and safer food production
- Food process engineering and non-thermal food processing technologies
- Reduce food loss and postharvest technology and management
- Smart food and beverage packaging system

ANNEX-2: AGENDA

Wednesday 12th September	
8:00 - 9:00	Registration and Networking
9:00 - 9:10	Introduction and Objectives of the Conference **Dr. Anil Kumar Anal, Asian Institute of Technology, Thailand**
9:10 - 9:15	Welcome Remarks **Dr. Gerhard Schleining, BOKU University, Austria**
9:15 – 9:20	Opening Remarks **Prof. Rajendra P. Shrestha** **Dean, School of Environment, Resources and Development,** **Asian Institute of Technology, Thailand**
9:20 – 9:25	Opening Remarks **Prof. Paola Pittia** **Chairperson, ISEKI**
9:25 – 9:30	Welcome Remarks **Representative of the French Embassy Bangkok**
9:30 – 9:35	Welcome Remarks **Representative of the German Embassy Bangkok**
9:35 – 9:40	Welcome Remarks **Representative of the Austrian Embassy Bangkok**
9:40 – 10:00	SEA-ABT Project Activities Dissemination **Dr. Gerhard Schleining, BOKU, Austria** **Dr. Sasitron Tongchitpakdee, Kasetsart University, Thailand**
10:00 – 10:20	ASIFOOD Project Dissemination **Dr. Laurent Roy, SupAgro, France** **Dr. Anil Kumar Anal, Asian Institute of Technology, Thailand**
10:20-10:40	**Coffee break**
10:40 – 11:00	Potential savings in CIP of food production plants through Hygienic Design, **Dr. Knuth Lorenzen** **EHEDG, Germany**
11:00-12:30	STAKEHOLDER ROUNDTABLE **(Moderators: Dr. Sasitorn T., Dr. Gerhard S., Dr. Laurent R. and Dr. Warapa M.)**
12:30-14:00	**Lunch Break**

Workshop: Sugars and Sweeteners in Beverages: Trends and Challenges	
14:00-14:15	Introduction and Background Information **Prof. Paola Pittia, University of Teramo, Italy** **Dr. Sasitorn Tongchitpakdee, Kasetsart University, Thailand**
14:15-14:45	Sugar Tax Insight, **Ms Natthakorn Utensute**, **Director of Tax Planning Bureau, Thailand**
14:45-15:15	Technological role of sugars and saccharides in beverages **Prof. Paola Pittia, University of Teramo, Italy**

15:15-15:45 Coffee break

15:45-16:15 Modern analytical tools for evaluation sugars and sweeteners
Dr. Dario Compagnone,
University of Teramo, Italy

16:15-16:45 **Update on sweetener applications for beverages**
Virat Chatnithikul, Managing Director, Scent Cottage Co. Ltd.,
Kanda Wanichkanjanakul, SEA Sales Manager, Mafco Worldwide

17:30-19:30 Welcome Cocktail in Hotel Windsor (Open for all participants and guests)

Thursday 13th September

08:00-9:00 Networking

8:45-09:00 **Opening and Briefing**
Dr. Anil Kumar Anal, Asian Institute of Technology, Thailand

Session 1: Innovative Food Ingredients and Food Quality

09:00-09:20 Innovative encapsulation strategies of bioactives in glassy matrices
Paola PITTIA, University of Teramo (Italy)

09:20-09:40 Effect of disaccharides on physical properties of rice starch edible film
Thongkorn PLOYPETCHARA, Ehime University (Japan)

09:40-10:00 Sequential Extraction of Hyaluronic Acid and Collagen from Chicken Eggshell Membrane
Nina Karla ALPARCE-JAIM, Asian Institute of Technology (Thailand)

10:00-10:20 Enzymatic Processing and Spray Drying of Honey Jackfruit (Artocarpus Heterophyllus) Powder
Chen Wai WONG, UCSI University (Malaysia)

10:20-10:40 Coffee break

Session 2: Functional Foods and Beverages

10:40-11:00 Stability of fortified infant formulas in the real food system
Sylvıe AVALLONE, MONTPELLIER SUPAGRO (France)

11:00-11:20 Bio-prospecting of riboflavin producing Lactobacillus strains of indigenous origin
Vijendra MISHRA, NIFTEM (India)

11:20-11:40 Quality Evaluation of Maltose Syrup Produced from Hydrolysis of Cassava (Manihot esculenta Crantz) Starch Using Malted Rice (Oryza sativa L.)
Theavy SREY, Royal University of Agriculture (Cambodia)

11:40-12:00 Extraction of Anthocyanin from Mulberry Residue Using Aqueous Two-Phase System
Minh Xuan Hong NGUYEN, Nong Lam University (Vietnam)

12:00-12:20 Status and prospects of Nepalese traditional foods and beverages
Anuj NIROULA, **National College of Food Science and Technology (Nepal)**

12:20-12:40 Physicochemical and functional properties of raw and processed moth bean (Vigna aconitifolia)
Seema MEDHE, Asian Institute of Technology (Thailand)

12:40-13:00 Extraction and in vitro evaluation of antidiabetic efficacy of bioactive extracts from okra (*Abelmoschus esculentus*)
Manisha Anand, Nutrichefs, Gurugram (India)

13:00-14:00 Lunch Break

13:30-14:00 POSTER PRESENTATION

Session 3: Food Loss Reduction and Postharvest Technology and Management

14:00-14:20 The use of predictive models in the context of food spoilage: the case of white pudding
Nicolas KORSAK, University of Liège (Belgium)

14:20-14:40 Application of Moringa oleifera leaves powder to extend the storage life of tilapia (Oreochromis niloticus) fish
Rakesh Rahul JADHAV, Asian Institute of Technology (Thailand)

14:40-15:00 Effects of Drying Temperature on Color and Total Curcuminoid Contents in Turmeric
Nilobon KOMONSING, Silpakorn University (Thailand)

15:00-15:20 Simultaneous Vacuum Falling Film and Rotary Evaporation for Producing Sweet Sorghum Syrup
Nur ISTIANAH, Brawijaya University (Indonesia)

15:20-15:40 Coffee break

Session 4: Food Safety and Risk Assessment Analysis in Food Production and Food Supply Chain Systems

15:40-16:00 Nanostructured electrochemical sensors for the rapid detection of pesticides
Dario COMPAGNONE, University of Teramo (Italy)

16:00-16:20 Development of wood packaging box containing bergamot oil to control of gray mold in strawberries
Saifon PHOTHISUWAN, Walailak University (Thailand)

16:20-16:40 Effect of lime oil vapor against L. monocytogenes during cold storage
Prangthip PARICHANON, Walailak University (Thailand)

16:40-17:00 Awarding and Closing Ceremony
Dr. Anil and Dr. Gerhard

ANNEX-3: ORGANISING INSTITUTIONS

Asian Institute of Technology (AIT)

The Asian Institute of Technology promotes technological change and sustainable development in the Asian-Pacific region through higher education, research and outreach. Established in Bangkok in 1959, AIT has become a leading regional postgraduate institution and is actively working with public and private sector partners throughout the region and with some of the top universities in the world.

58 Moo 9, Km. 42, Paholyothin Highway,
Klong Luang, Pathum Thani 12120, Thailand
https://www.ait.ac.th/

ISEKI-Food Association (IFA)

IFA is an independent European non-profit organisation, established in 2005 by university institutions, research institutes, companies and associations related to food, coming from all over the world.
The IFA **mission** is to support

- **Lifelong learning in the food sector**, encompassing both **academia & industry**
- **Teachers** and **Trainers** to improve efficacy of teaching
- **Students** to gain knowledge more easily
- **Industry staff / Food professionals** to make use of research results
- **Researchers** to facilitate collaboration

Muthgasse 18, 1190 Vienna, Austria
https://www.iseki-food.net

ANNEX-4: SPONSORS

The 1st International IFIFS 2018 Conference has been supported by the following sponsors:

ENDORSERS

ICC-Interantional Association for Cereal Science and Technology

ISEKI-Food Association

BOKU

King Mongkut University

Kasetsart University

Chulalongkorn University

University of Teramo

Supagro